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THE AMERICAN JOURNAL OF PHYSIOLOGY

EDITED FOR

THE AMERICAN PHYSIOLOGICAL SOCIETY

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VOL. XCIV—NO. 3

Issued September 1, 1930

BALTIMORE, U. S. A.

1930

Entered as second-class matter, August 18, 1914, at the Post Office at Baltimore, Md., under the Act of March 3, 1879. Acceptance for mailing at special rate of postage provided for in section 1103, Act of October 3, 1917. Authorized on July 5, 1918

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VOL. 94 SEPTEMBER 1, 1930 No. 3

AN INVESTIGATION OF THE PHYSICAL STRUCTURE OF FIBRIN BY X-RAY CRYSTAL METHODS

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Received for publication April 7, 1930

There are two views regarding the structure of fibrin gel: one, that fibrin is deposited as an interlacing meshwork of needle-like crystals, the other that the gel is particulate in origin being formed by the agglutination of submicroscopic particles into threads or fibrils. Readers interested in the development of the first theory may consult the papers by Schimmelbusch (1), Stübel (2, 5) and Howell (3, 4). The second theory is set forth in papers by Mayer (6), Cesana (7) Hekma (8, 11), Barratt (9) and Kugelmass (10).

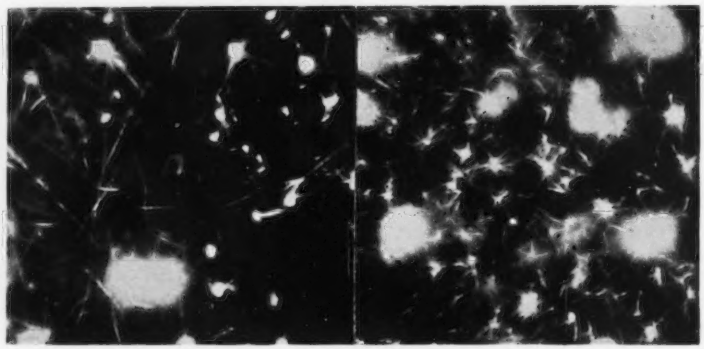
In previous work, conclusions as to the physical structure of the fibrin gel have been drawn from the microscopic appearance, or from considerations of colloidal behavior. The most rigid test, however, which may be used to tell whether fibrin is crystallin, is one which can demonstrate that the atoms or molecules are arranged with a definite spatial symmetry. That is, if the atoms of fibrin form a space-lattice, then we may regard it as having fulfilled the most exacting requirement of crystallinity. X-ray diffraction pattern methods furnish us with the necessary means of making such determinations. (See Bragg and Bragg, *X-rays and crystal structure*, 4th ed., G. Bell & Sons, Ltd., London 1924.)

In the experiments reported in this paper the powder method of x-ray spectrography was employed to study the structure of fibrin. The current to the x-ray tube was rectified by a synchronous disc-rectifier and in all experiments the tube current was 20 milli-amperes at 40 kilovolts. The tube was a Coolidge water cooled tube with a molybdenum target. A zirconium oxide filter removed practically all wave lengths but the K_α doublet; and, since this was not resolved, the mean wave length (12) of 0.71Å was used in the calculation of spacings.

As a preliminary experiment, fibrin formation was studied micro-

scopically using dark field illumination. Oxalated centrifuged beef blood was used, fibrin formation being induced by adding dilute calcium chloride solution.

In the main, observations of other workers were confirmed. The fibrin needles appeared to result from the union of every minute particles. These coalesced, forming long slender structures, which at first seemed to consist of minute particles. After a time, the union between the particles became more intimate, and long slender needle-shaped structures, resembling fine crystals, were produced. Figures 1 and 2 are photomicrographs of the fibrin needles. In figure 1 the concentration of calcium was low and coagulation was slow. The needles of figure 2 result from higher concentrations of calcium and more rapid clotting.



Figs. 1 and 2. So-called "crystals" of fibrin from oxalated beef plasma. (× 750)

In the first experiment, oxalated plasma was made to clot by adding calcium chloride. Microscopic examination of samples of the clot showed it to be composed of an interlacing meshwork of needles. After the clot had contracted, it was broken up and washed several hours in tap water, rinsed in distilled water, partially dried between filter paper, and placed in a desiccator. After thorough drying, the fibrin was finely ground so as to pass through a 200 mesh sieve and packed as a pellet in a specimen holder. The pellet was 3 mm. thick. An exposure of an hour and a half resulted in a diffraction pattern. This demonstrated that either the fibrin was crystallin or had associated with it a crystallin substance not removable by washing in water.

In the second experiment beef blood was allowed to clot normally. The fibrin was washed, dried, ground, and x-ray exposures made as before but no diffraction pattern resulted.

After many attempts to find the source of the spectrum in experiment one

it was discovered that if solutions of both calcium chloride and potassium oxalate were added to finely powdered fibrin prepared as in experiment two the diffraction pattern could be reproduced. Neither calcium chloride nor potassium oxalate alone is effective. The spectrum of this material is not that of pure calcium oxalate, as was shown by comparing it with a diffraction pattern of pure calcium oxalate. Table 1 gives the spacings in Ångström units, calculated from the diffraction pattern of the crystallin material in this fibrin-calcium oxalate complex, as compared with those of pure calcium oxalate.

When blood is oxalated, calcium oxalate is precipitated and can be centrifuged out. The x-ray diffraction pattern given by this material was found to be identical with that of pure calcium oxalate. In plasma

TABLE 1

Comparison of intensities and lattice spacings of the diffraction patterns of the fibrin-calcium oxalate complex and of pure calcium oxalate

FIBRIN-CALCIUM OXALATE			PURE CALCIUM OXALATE		
Line number	Relative intensity	Spacing	Line number	Relative intensity	Spacing
		Ångström units			*Ångström units*
1	Intense	6.29	1	Intense	6.07
2	Medium	4.54	2	Intense	3.70
3	Medium	3.70	3	Intense	3.02
4	Faint	3.20	4	Medium	2.54
5	Intense	2.82	5	Intense	2.38
6	Faint	2.42	6	Faint	2.28
7	Medium	2.28	7	Faint	2.11
8	Faint	2.24	8	Medium	1.98
9	Faint	1.99	9	Faint	1.84
10	Faint	1.92	10	Faint	1.75
11	Faint	1.87			

thus freed of calcium and having an excess of oxalate, fibrin formation may be induced by the addition of calcium chloride. The fibrin formed in this manner gives a diffraction pattern like that in experiment one. This experiment indicates that it is the formation of calcium oxalate in the presence of fibrin, and not fibrin itself, which is responsible for the diffraction pattern. Some crystals, therefore, must be formed which involve calcium and oxalate radicals, and possibly fibrin.

There is no evidence that the fibrin itself is crystallin under these conditions. The aberration in the crystal structure of calcium oxalate can be caused by adding to fibrin from a normal clot, which itself will give no diffraction pattern, potassium oxalate and calcium chloride, that is, by forming calcium oxalate in the presence of fibrin. Under either of these conditions the crystal form of calcium oxalate appears to have been

modified by some intimate association with fibrin, and this association cannot be broken by washing.

Since this investigation sought only to determine whether fibrin be crystallin or non-crystallin no attempt was made to identify this substance more completely. However, it appears probable that the formation of an insoluble calcium salt is essential. The following experiment supports this hypothesis. Citrated plasma was clotted by diluting with water and adding calcium chloride. Since calcium citrate is relatively water soluble it seemed probable that fibrin could be washed free of calcium citrate. As expected, fibrin thus prepared gives no diffraction pattern.

SUMMARY AND CONCLUSIONS

Experiments were undertaken to answer the question: Does the fibrin of a fibrin gel exist in a crystallin condition? The x-ray diffraction pattern method was used to answer this question because it furnishes the most rigid test for crystallinity.

The fibrin of a normal blood clot gives no diffraction pattern.

"Fibrin" formed by adding calcium chloride to oxalated plasma gives a diffraction pattern. This pattern can be caused by adding calcium chloride and potassium oxalate to dry powdered fibrin from a normal clot which itself gives no diffraction pattern.

Fibrin prepared by adding calcium chloride to citrated plasma gives no diffraction pattern.

The experiments clearly show that the fibrin, whether it be taken from a normal blood clot or from a clot formed by adding calcium chloride to citrated or oxalated plasma, has not itself a definite space lattice, although under certain conditions crystallin material may be associated with it.

Fibrin, therefore, is not a crystallin substance.

I wish to express my thanks to Prof. S. C. Brooks for his helpful criticisms, and to Mr. J. E. Gullberg for the photomicrographs and assistance in the dark-field microscopy.

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STUDIES ON THE INNERVATION OF THE LARGE BOWEL

II. The Influence of the Lumbar Colonic Nerves on the Distal Part of the Colon

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Received for publication April 9, 1930

In a previous paper (1) we have shown that some of the fibers of the lumbar colonic nerve are concerned with the motor innervation of the internal sphincter of the anus. In this paper we shall record our experiments to show the part played by the lumbar colonic nerve in the innervation of the distal part of the colon. This nerve arises from the inferior mesenteric ganglion, the roots of which have two immediate sources: the lumbar sympathetic chain (medullated fibers), and the pre-aortic plexus (non-medullated fibers derived from the celiac and semilunar ganglions). Thus the thoracic splanchnic nerves play a part in the innervation of the bowel as far as the distal colon, and they are reinforced by fibers leaving the great sympathetic chains at lower levels. The lumbar colonic nerve of the dog is well adapted for such experiments. At its origin from the inferior mesenteric ganglion it forms a compact strand, readily accessible for surgical interference; moreover, by dividing it close to the ganglion one can be reasonably sure of interrupting all the fibers of the thoracicolumbar outflow which pass to the distal part of the colon, although at each experiment careful search must be made for the fine filaments which the nerve occasionally receives 1 or 2 cm. from its source.

Method of experiment. Adult dogs, weighing about 10 kgm., were used in the experiments. The colon was emptied by an enema on the morning of the operation. The dogs were anesthetized by the intravenous injection of sodium iso-amylethyl barbituric acid, 25 to 35 mgm. for each kilogram of body weight. The abdomen was opened in the middle line, and the descending and pelvic parts of the colon were identified. A loop of bowel about 10 cm. long was isolated between two ligatures, in such a way that the main trunk of the inferior mesenteric artery reached the loop at about its middle. There is no danger of interfering with the vitality of such a loop if care is taken to place the ligatures between the terminal arcades of the vessels and the wall of the bowel. A metal cannula was inserted into the colon, through its antimesenteric border, and was retained in position by two pursestring sutures. The lumbar colonic nerve was

freed from the inferior mesenteric artery for a short distance close to the inferior mesenteric ganglion, and stimulating electrodes, shielded from the neighboring structures by a small piece of rubber tissue, were placed in position on it. The loop of bowel was covered with warm moist towels.

In such a preparation as this, the colon may be influenced by its intrinsic nerve supply and by the thoracicolumbar sympathetic fibers reaching it along the lumbar colonic nerve. The motor fibers from the pelvic nerves reach the more proximal parts of the colon by way of the wall of the bowel; in our preparations these fibers were prevented, by the distal ligature, from acting on the isolated loop.

The cannula in the colon was led directly to a cylinder connected to a piston recorder. The fluid used in the system was a physiologic solution of cane sugar; since sucrose is not absorbed by the colon, the volume of fluid in the system remained constant over a long period.

EFFECT OF STIMULATION OF THE LUMBAR COLONIC NERVE. It is well known that faradic stimulation of the lumbar colonic nerve causes relaxation of the large bowel. The latent period is extremely short, and the fall in intracolonic pressure is rapid. The same effects may be produced by stimulating the "roots" of the inferior mesenteric ganglion, whether derived from the lumbar sympathetic chain or from the pre-aortic plexus. We happened by chance on a curious limitation of the amount of dilatation which could be produced in the loop of bowel. It has been shown previously that the lumbar colonic nerve shows little, if any, fatigue on repeated stimulation (2). We found, however, that with a given "head" of fluid, there came a point when stimulation of the nerve did not cause further dilatation of the bowel; on the addition of a further small quantity of fluid, stimulation of the nerve caused immediate dilatation of the bowel (fig. 1). This process of alternate addition of fluid and stimulation of nerve could be repeated until high degrees of dilatation of the bowel were obtained. These observations suggest to us that for every given volume of intestinal content, there is an optimal intracolonic pressure; in other words, that one function of the thoracicolumbar sympathetic nerve supply to the colon is the regulation of suitable plastic tonus in the wall of the bowel.

IMMEDIATE EFFECT OF SECTION OF THE LUMBAR COLONIC NERVE. To investigate this point, a more elaborate preparation was used. The segment of colon was prepared as before. The lumbar colonic nerve was taken up in the loop of a wire snare carried in a fine metal tube, and the abdominal wall was closed save for the exit of the colonic cannula and snare. A tracheal cannula attached to a long tube was inserted, and the whole animal was immersed in a tank containing Ringer-Locke solution; the tank was kept at a constant temperature of 40°C. by means of a thermostat. It was found necessary to modify the recording system, since the colonic contractions after the neurectomy became too vigorous to be

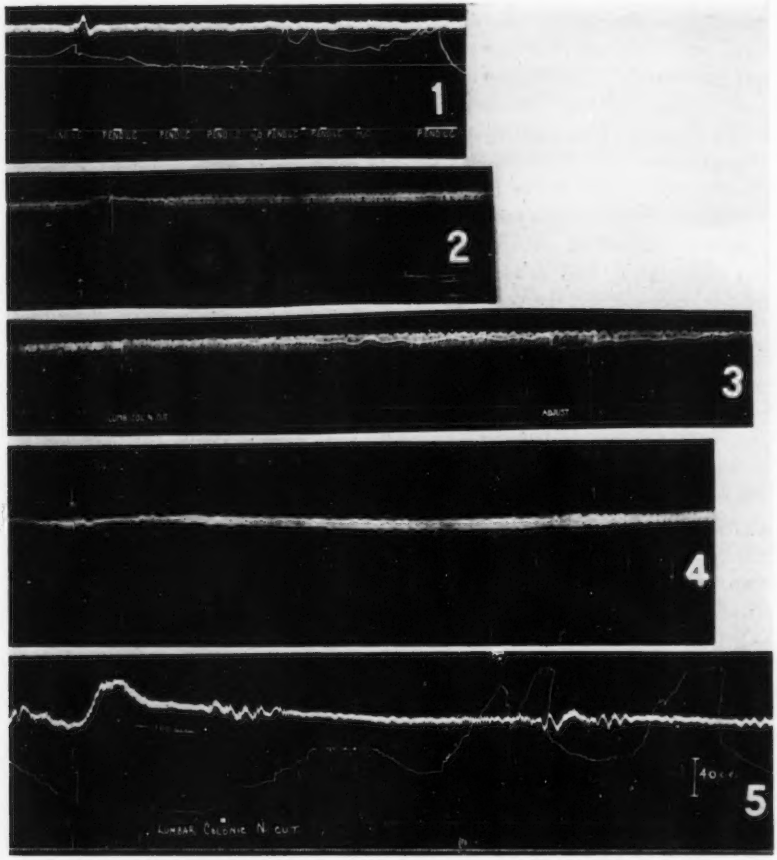

Fig. 1. Absence of colonic dilatation on stimulation of the peripheral end of the cut lumbar colonic nerve, until the volume of fluid in the recording system was increased. Time marker set at five seconds.

Fig. 2. Effect of dividing the lumbar colonic nerve slowly. Preliminary inhibition, followed by marked rise in intracolonic pressure. Time marker set at five seconds.

Fig. 3. Immediate rise of intracolonic pressure, and cessation of peristalsis, after section of the lumbar colonic nerve. Time marker set at five seconds.

Fig. 4. Markedly increased peristalsis after division of the lumbar colonic nerve. The figures above each contraction represent the number of cubic centimeters of fluid in excess of the capacity of the recording system. Time marker set at five seconds.

Fig. 5. The loop was allowed to remain under the influence of the pelvic nerves. Marked increase in both intracolonic tension and peristalsis after division of the lumbar colonic nerve. Time marker set at five seconds.

recorded on the drum. A "shunt" was therefore introduced, and only a small but constant proportion of the changes in the intracolonic pressure was registered. The loop of colon was first allowed to record for about an hour. The tracings obtained during this period were divisible into two broad groups: those in which the colon was active, and those in which it was quiescent. Whatever the nature of the control tracing, the division of the lumbar colonic nerve by the wire snare always led to an immediate increase in intracolonic pressure. The effect of dividing the nerve slowly is shown in figure 2. While the nerve was being crushed by the snare it was stimulated, and dilatation of the colon resulted; as soon as it was crushed completely, the pressure in the intestine rose steeply.

In certain cases (fig. 3) active peristalsis ceased after section of the nerve, and the loop became contracted. In other cases (fig. 4) neurectomy was followed not only by an increase in the intracolonic tension, but also by a greatly increased amplitude of the peristaltic contractions. These were maintained for at least one and a fourth to two hours.

It may be noted that these increased contractions occurred in a loop of colon cut off from its motor nerve supply; this affords further proof of the high degree of autonomy possessed by the plain muscle of the bowel and its intrinsic nerves. In our opinion the immediate and unvarying effect of neurectomy must mean that the inhibitory nerves to the bowel are constantly in action, whether they are adjusting the caliber of the bowel to varying volumes, or maintaining it to contain a constant volume.

In another series of experiments the pelvic nerves were allowed to act on the preparation by placing one ligature on the sigmoid colon, and leading the recording tube from the anus, where it was fastened by a pursestring suture. The pelvic nerves were then free to ascend in the wall of the rectum and colon to their distribution. Under these conditions similar results followed section of the lumbar colonic nerve; the peristaltic contractions of the bowel were greater than when these nerves were excluded from the preparation (fig. 5).

SUMMARY AND CONCLUSIONS

1. A series of experiments on the influence of the lumbar colonic nerve on the distal part of the colon is described.

2. The lumbar colonic nerve exerts a constant inhibitory influence on the musculature of the distal part of the colon.

3. Section of the lumbar colonic nerve invariably leads to an immediate increase in intracolonic pressure.

4. Section of the lumbar colonic nerve may lead to an increase in the amplitude of the contractions of the colon.

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MECHANISM OF TEMPERATURE SENSATIONS[1]

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Received for publication April 11, 1930

In his famous treatise (1846) E. H. Weber (1) describes some experiments easy to repeat, showing that the so-called temperature sensations are not determined by the actual temperature of the skin but by its rising or falling. But he himself records an observation not agreeing as it seems with this conception. He put a key cooled down to the temperature of melting ice for 30 seconds on his forehead and felt cold not only during the period of application but also some twenty seconds afterwards. As the skin, after taking off the key, will be warmed up by the blood stream a feeling of warmth ought to be expected. Weber presumes that the skin surrounding the cooled area must be made responsible for it since it gives off heat and cools accordingly.

E. Hering (2) confirmed in 1877 Weber's observation but, regarding the latter's explanation unsatisfactory, developed the well-known theory of his own.

There can be no doubt, that areas of the skin other than those actually touched by the key are cooled off by it. Thomas Lewis has shown by measuring the temperature of the skin with thermocouples, that from a hand put in cold water the low temperature of the venous blood can be traced up to the shoulder. There must be also a conduction of heat from the surrounding skin toward the cooled area. Thus Weber's explanation may be right at least for a part of the phenomenon and it seems worth while to formulate an experiment designed to give more precise information.

To this end I cooled the skin with a Thunberg temperator of 2 sq. cm. area kept at the temperature of 5°C. The temperator fitted exactly in a hollow ring through which water of normal skin temperature was flowing, that is, water which was felt neither warm nor cold. The hollow ring was set on the skin first to let thermic sensations, if initially present, die out. Then the temperator was introduced causing a strong sensation of cold diminishing markedly in intensity during the first half-minute. When now the temperator was taken away, the ring remaining at its place, the sensation changed in one or the other of two ways: Either the sensation of cool or

[1] Presented at the xiii International Physiological Congress, Boston, Mass., August 22, 1929.

cold simply dropped off being replaced by an indifferent feeling; or a weak and fleeting sensation of warmth appeared.

In testing the skin at the places designed for the experiment for spots sensitive towards small sized thermic stimuli just above liminal strength it turned out that the after-effect of cooling was determined by the presence or absence of warm spots beneath the temperator. In places where they were present the removal of the temperator was followed by a sensation of warmth, slowly setting in and never reaching much intensity. In all other places the sensation of cool or cold vanished at the moment the temperator was taken off, no after-effect coming on.

It was shown by H. Rein (3) in 1925 that the warm spots are distributed very scantily in comparison with other kinds of sensitive spots. On the trunk and limbs one finds generally not more than 0.3 per sq. cm., or 1 in 3 sq. cm. In using a temperator of 2 sq. cm. or a similar cooling device the probability of getting a sensation of warmth is very small. The cold spots on the other hand are relatively numerous and when no precaution is taken against the conduction of heat from the neighbourhood toward the cooled area the cold spots of the former will be irritated. That is what Weber and Hering found.

It seems, therefore, that the explanation Weber has given of the experiment in question is fully justified and there is no necessity of modifying it in the way Hering has proposed. At the same time it is clear, that the conception Hering has developed of what he calls adaptation of the temperature sense, representing as it were a sort of nervous rebound against the irritating effects of any temperature different from the normal one, is no longer necessary, the physical accommodation of the skin to the external temperature being apparently by itself sufficient to remove the stimulating effects of the latter.

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THE CIRCULATORY REACTION TO GRAVITY IN HEALTHY YOUNG WOMEN

Evidence Regarding its Precision and its Instability

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Received for publication April 14, 1930

Previous studies have shown that in quiet standing healthy young women make extensive circulatory adjustments which are maintained, as the quiet erect position is prolonged, with a marked variation in success in different individuals. In some cases the adjustment first made on standing shows almost no change during a fifteen minute standing period, in other successive readjustments may entirely fail and there may be extreme dizziness and fainting after a period as short as six or eight minutes. However, the first adjustment tends to be of the same type in all cases, an increase in the rate of the heart accompanied by a rise of diastolic pressure and a narrowing of the pulse pressure. The systolic pressure shows no extensive change; it may rise or fall or be unchanged. The narrowing of the pulse pressure is of sufficient magnitude to suggest a much decreased output per beat for which the faster rate of the heart may not be able to compensate and thus to maintain the reclining circulatory minute volume. Recent studies at the Hopkins laboratory, however, (Grollman, 1928) have indicated that the reclining circulatory minute volume is maintained during standing. Other recent studies (Thompson, Thompson and Dailey, 1928; Thompson, Alper and Thompson, 1928) have given further evidence in accord with the earlier studies of the present series (Turner, 1927a, 1927b, 1929) and with those of Field and Bock, 1925; Lawrence, Hurxthal and Bock, 1927; Collett and Liljestrand, 1924, namely, that there is a general tendency in standing toward reduction of the circulatory minute volume as quiet standing is prolonged. Such a reduction is obviously in opposition to the needs of the occasion and yet it is in harmony with many phenomena in every-day life in which circulatory embarrassment occurs during quiet standing. In view of the discordant evidence it seemed desirable to continue the study further.

THE RELATION BETWEEN THE METABOLIC NEED AND THE CIRCULATORY ADJUSTMENT SEEN IN THE ERECT POSITION. It was necessary to find out what part of the circulatory adjustment in standing is due to the work of

standing considered as exercise. Various studies (Benedict and Benedict, 1924; Turner, 1927b; Bainbridge, 1923, many experiments summarized) show the rise in oxygen consumption for standing as compared with reclining to be about 10 to 20 per cent, with a rise in heart rate as great as that observed for exercise by walking, etc., which raises the oxygen consumption by as much as 200 per cent. The pulse pressure in exercise is widened, mainly by a rise of the systolic pressure; in standing the pulse pressure is narrowed due to the rise of diastolic pressure. In exercise the factors making for the return of blood to the heart are in evidence, augmented respiration, muscular movements, while in quiet standing these afford almost no help. The question thus arises, may we not in considering the circulatory adjustment for quiet standing neglect the exercise factor?

Helmreich, 1923, made suggestive studies on a few children by placing them on a tilting board and determining their oxygen consumption at different angles, at the same time counting the pulse. He concluded that there are two kinds of pulse rise, a dynamic rise, corresponding to the increased use of oxygen seen in exercise, and a static rise, corresponding to the changed relation of the body to gravity. We extended this type of procedure to a larger group of young women and included pressure determinations.

Through the courtesy of the Department of Physiology in the Harvard School of Public Health a tilting board was made available. This board by chains below can be tilted to any angle and held there. There are adjustable head, foot and shoulder rests. The comfort of the board is increased by a pad, a very low pillow for the head, and if the subject wishes it another little pillow to lessen the rigidity of the knees. All the subjects were healthy young women, college students, young assistants and the like, in age ranging from 16 to 30 years. For series I, done by Isabel Newton at Mount Holyoke College, the experiments began by two basal metabolism determinations made with the usual precautions During the six minute period of each test three counts of the pulse alternated with three determinations of arterial pressures. A Benedict-Roth metabolism apparatus was used with an electric blower in the circuit to facilitate air movement through the elongated tubes made necessary by the tilting board. After a brief rest, the board was tilted to an angle of 62°, chosen as about the steepest angle at which the subject feels she is entirely held up by the board. As far as work goes she is reclining, but the gravity problem of the circulation approaches that of standing. Metabolism and circulatory tests were repeated. After another horizontal rest period the subject was tilted to the erect position, took one step off the board so that she was not supported by it, and repeated the experimental procedure. On different days the order of tests for 62° and 90° was reversed. A consistent effort was made to have the subjects both mentally and physically at ease.

The results of these tests in series I, 31 experiments on 12 subjects, showed a rise in oxygen consumption for the 62° position as compared with reclining of only 5.8 per cent, for standing, of 19.0 per cent. The circulatory findings are given in table 1. Systolic and diastolic figures are shown only by averages in a footnote to the table since they showed so little of interest. It will be seen that while the oxygen consumption changed an almost negligible amount for both 62° and standing the circulatory changes

TABLE 1

Heart rates and pulse pressures of experiments of series I, with metabolism tests, 31 experiments on 12 subjects; also of series II, without metabolism tests, 26 experiments on 12 subjects

Maximum and minimum figures show the range covered, but most of the determinations were near the average figures. Rates per minute, pressures in mm. Hg.

	HEART RATES					PULSE PRESSURES				
	Basal reclining	Tilted 60–62°	Per cent rise due to tilt	Standing or 90°	Per cent rise due to vertical	Basal reclining	Tilted 60–62°	Per cent fall due to tilt	Standing or 90°	Per cent fall due to vertical
Series I:										
Maximum	81.2	102.1	48.3	101.1	50.0	53.55	42.5	39.8	43.5	36.2
Minimum	56.5	73.2	13.8	74.1	14.2	34.7	24.7	5.2	23.65	3.2
Average	66.2	85.8	30.0	84.35	27.8	41.1	32.8	20.2	31.4	23.6
Series II:										
Maximum	70.0	98.2	70.8	108.9	89.3	51.75	38.3	44.1	31.1	44.8
Minimum	51.5	68.3	26.5	71.6	29.5	26.0	17.6	3.0	15.2	5.0
Average	59.1	83.4	41.1	89.1	50.8	38.2	27.6	27.6	25.6	33.0

The averages for systolic and diastolic pressures were:

Series I:

Systolic reclining, 111.1; tilted to 62°, 111.9; standing, 110.9.
Diastolic reclining, 70.0; tilted to 62°, 79.7; standing, 79.8.

Series II:

Systolic reclining, 109.4; tilted to 60°, 108.6; tilted to 90°, 109.0.
Diastolic reclining, 72.05; tilted to 60°, 80.9; tilted to 90°, 83.3.

were very marked and almost the same for the two positions. The experiments showed the usual variations for the prolonged erect positions: some subjects with an even and steady adjustment, others changing progressively as time went on. One subject became so dizzy and faint on two occasions that the experiments had to be cut short. Her results on these days could not be included in the averages.

We seem thus to be justified in separating the gravity reaction from the exercise reaction as far as the circulation is concerned. But any experi-

ments for a human subject are prone in themselves to lead to circulatory variations; therefore it seemed advisable to run the simplest possible control series in which no metabolism determinations were made but only circulatory data were gotton for the tilted positions. Series II, then, was run by Abby Turner at Woods Hole as follows. The subjects came to the laboratory at least three hours after their last meal, with no strenuous exercise in the interim. The time of day was always the same for a given subject. After a preliminary rest of ten or fifteen minutes on the board, the subject's reclining pulse and pressure determinations were made in alternate minutes until they showed a fairly steady state, usually in six or eight minutes. She was then tilted to 60° or to 90° and pulse and pressure determinations went on in alternate minutes for a period of fifteen minutes. She was returned to the horizontal for a control period of six or eight minutes and then tilted to the angle not used before and observed for another fifteen minutes. She did not get off the board at all during the experiment and the whole program was kept as free from minor interruptions as possible.

The results of series II, 26 experiments on 12 subjects, are also included in table 1. A single experiment is shown in figure 1, to give the nature of the changes observed as an experiment went on. According to our custom, in neither series I nor series II were any results of a first day with a subject included unless they were indistinguishable from later results. It will be noted that while the results of series II are in general like those of series I there is a perceptible difference between the findings for 60° and for 90°. The difference in experimental procedure meant that the subject did *less* work in series II than in series I at 90°, yet the rise in heart rate is greater and the narrowing of pulse pressure more marked than in the first series. In series II the effect of gravity is less disturbed by muscular movements which might help the blood back to the heart—this seems the probable explanation of the difference between the two series. This explanation is supported by two episodes in individual experiments in which slight movements led to a slowing of the heart or to an increase of pulse pressure.

NICETY OF ADJUSTMENT TO GRAVITY. Since in series II there had been a perceptible difference between the responses to tilting to 60° and to 90°, the differential response to tilting to different angles was studied more carefully. This series, III, consisted of 17 experiments on 11 subjects all but two of whom had been subjects in the preceding series. The subject rested on the board for the usual preliminary period. Her reclining rates and pressures were then determined alternately for six minutes. She was then tilted for six minute periods to each of the following positions, 90°, horizontal, 15°, horizontal, 30°, horizontal, 45°, horizontal, 60°, horizontal, 75°, horizontal, 90°. The subject remained as quiet as possible during the whole test since even slight movements affected the findings noticeably. The use of 90° at both beginning and end checked the steadiness of her

state during the whole experiment. Each experiment was run through the whole set of angles unless the subject became restless or fatigued, in which case work with her was impossible. The best subjects were those for whom relaxation seemed easy.

There was for each angle a different response, the circulatory apparatus thus indicating its ability neatly to discriminate angles as close together as

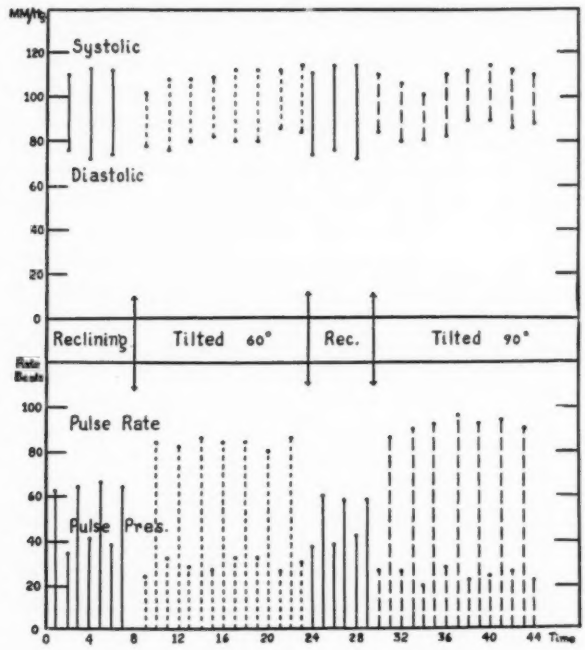

Fig. 1. Effect of tilting to 60° and to 90° for fifteen minutes each. Single experiment from series II, all findings. Upper part of chart shows arterial pressures, lower part pulse rates and pulse pressures. Dotted lines, 60°; dash lines, 90°; continuous lines, reclining data. Time below in minutes from beginning of experiment. Preliminary rest of ten minutes not shown. Note that pulse rate increases and pulse pressure narrows more for 90° than for 60°. Middle reclining period used as control period.

15°. The average results, computed from the findings for minutes three to six of the periods concerned are shown in the graph in figure 2. Minutes 1 and 2 were omitted since they were the time of adjustment to the new position and showed a few irregularities, due apparently to slight movements as the new position was assumed. The lessened response in the last 90° period compared with what might have been expected from the slope of

the rest of the curve is probably not a true case of failure to respond to the last 15° in tilting, but due to the almost unavoidable movements made when the subject found herself supported by her own feet instead of by the board beneath her back. All subjects adjusted themselves slightly to balance better in the erect position even though asked to let the board hold them up as far as possible.

A few experiments were done to ascertain the effect of tilting head down. This is a much more difficult experiment since the position induces unfa-

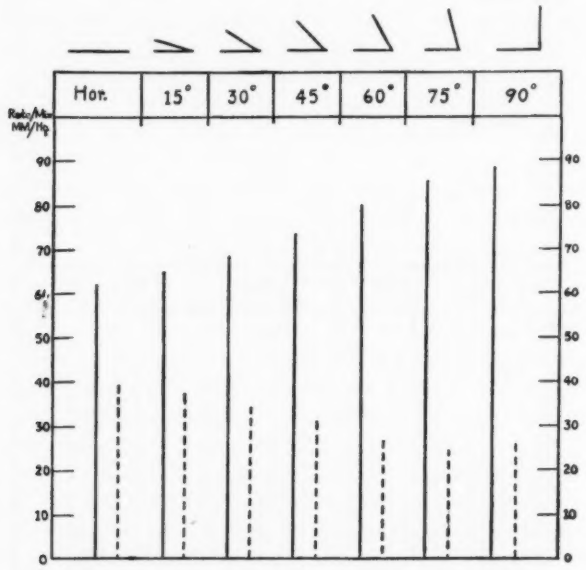

Fig. 2. Summary of results of tilting, head up, to angles indicated. Series III, 15 experiments on 11 subjects, 6 minutes in each position, with return to horizontal after tilting to each angle shown. Continuous lines, heart rates; dotted lines, pulse pressures. Note progressive rise of heart rate and progressive fall of pulse pressure. Systolic change was slight, diastolic pressure rose.

miliar sensations, sometimes slightly uncomfortable, and subjects are wont to show a more or less well-concealed state of apprehension. Considerable training is usually needed for valid results. The averages of the few experiments so far done indicate a progressive slowing of the heart as the head-down angle increases from 15° to 35°, and a slight widening of pulse pressure, in other words, an extension of the effect seen in the head-up position beyond what might be called the zero point of the horizontal. This series will be continued.

Parallel tilting experiments on men are in progress at the Harvard School of Public Health.

It is then apparent that the individual's first response to gravity when the position is changed from the horizontal reflects the difficulty with which the blood reaches the medullary centres. As time passes this adjustment tends to fail more or less and many persons have sought to determine the existence of stagnation below the heart when the person or animal is upright Among these may be mentioned Hill, 1895; Hill and Barnard, 1897; Sewall, 1916, 1919; Crampton, 1913, 1915; and more recently Atzler and Herbst, 1923; and Drury and Jones, 1927. The two last mentioned worked by studying the increase in the size of the leg and foot when immersed in water, as the person sat or stood. Drury and Jones worked particularly on the resulting edema, but in any case we see an accumulation of liquid of circulatory origin. To extend this work with emphasis on the accompanying heart and pressure findings the experiments of series IV were undertaken by Florence Haynes at Mount Holyoke College and continued by Abby Turner at Woods Hole as series V. The two series were treated separately because of the effect of temperature on the peripheral circulation—series IV was in winter, series V in summer.

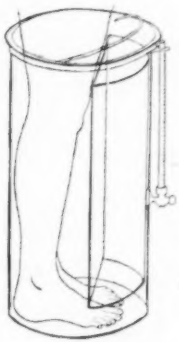

Fig. 3. Can with gauge, to show arrangement for determining increase in size of legs in prolonged standing. Inner can lessens volume of water concerned.

INCREASE IN THE VOLUME OF THE LEGS IN QUIET STANDING. The necessary preliminary experiments were these. It was ascertained that a subject could stand for a fifteen minute standing period in a large can of water, deep enough so that the water came well above the knees and of a temperature such that it felt neither warm nor cool to the skin, without perceptible variations in circulatory findings from those obtained during a similar period of standing in air as usual. To measure the increase in leg size, a can was used with an outside gauge and with a considerable portion of the inside space above the level of the feet cut out by an accessory can clamped into the larger can. The total volume of water surrounding the legs was thus made as small as was convenient so that any rise observed in the gauge might be over as wide a space as possible. See figure 3, in which the relation of the subject to the can is shown. A preliminary trial was made to determine the subject's leg volume at a given water level, to make a rough calibration for the rise of water, and to determine the temperature of the water which would feel at that time neither warm nor cool. After a rest of ten or fifteen minutes the test followed according to the usual technic of our standing procedure (Turner, 1927b) a preliminary reclining period,

a standing period of fifteen minutes in the can and finally a control reclining period, with pulse and pressure determinations in alternate minutes throughout. The level of water was read immediately after the subject stepped into the can. It began to rise with considerable speed in the first half-minute of standing, often more than 100 cc., and the rise continued, though more slowly, as standing was prolonged. There is no indication that it had gone to its highest point within the fifteen minutes of the experiment, indeed, Atzler says the rise continues for two hours at least. The water level was read again just before the subject stepped out of the can and the water drawn off by a siphon to the original level, thus giving a check on the reading of the scale by the measurement of the portion

TABLE 2

Changes in leg volume induced by standing for 15 minutes in a can of water of neutral temperature. Series IV, winter, 35 experiments on 20 subjects. Series V, summer, 6 experiments on 4 subjects

Many of the determinations are near the average value

	TEMPERATURE OF BATH	LEG VOLUME	INCREASE IN LEG VOLUME	PER CENT INCREASE IN LEG VOLUME
		cc.	cc.	
Series IV:				
Maximum	33.0	12,500	302	2.68
Minimum	28.0	6,750	90	1.19
Average	30.2	9,149	175	1.90
Series V:				
Maximum	31.65	9,355	317	4.10
Minimum	30.1	6,735	180	2.67
Average	31.15	7,260	256	3.36

drawn off. Sources of error in the method are the slight movements of the subject, especially those in the first and last half minutes of the standing period. The results of the 35 experiments on 20 subjects in series IV and those of 6 experiments on 4 subjects in series V are given in table 2.

The amount of liquid which collects in the legs, 175 cc. in series IV and 256 cc. in series V, is thus great enough to be significant, though the amount is not striking. By an arbitrarily chosen value of 65 cc. per kilogram of body weight (Chang and Harrop, 1927) the blood volumes of the 20 subjects of series IV were found to approximate 3800 cc. The average increase in leg size thus means the detaining in the part of the body concerned of about 4.6 per cent of the total blood in circulation, an amount which seems insufficient to account for the circulatory changes observed.

Whether the increase in leg size in standing is due to blood in the vessels or to lymph or tissue fluid is of interest. There are two indications of its being due in the main to blood, one the very rapid rise in the gauge at the beginning of the erect position and the equally rapid return of circulatory findings to their reclining level when the subject lies down after standing, the other the swift color change when the individual reclines. The legs and hands may have been distinctly purplish while the subject was standing, they become paler and pinkish almost instantly, a swiftness of reaction like the color change in the face when the head is put below the level of the heart.

CONTROL OF CIRCULATORY FINDINGS BY ABDOMINAL SUPPORT. If the blood tends to stagnate in the abdominal region during standing a firm support to supplement that given by the muscles of the abdominal wall ought to help in preventing this stagnation. This is of course the basis of frequent clinical prescription. To determine the extent of this effect on the circulation the experiments of series VI were undertaken by Florence Haynes at Mount Holyoke and extended as series VII by Abby Turner at Woods Hole.

The subject had as a control an ordinary standing test (Turner, 1927b) with no abdominal support. She repeated this test with the support of either a corset or a firm abdominal bandange. The experiments of series VI include several types of support, none entirely free from the danger of slipping when the subject rose or reclined. The experiments of series VII were all done with a broad ACE bandage which was easily adjustable and never slipped. In every experiment of both series there was a distinct effect as compared with the control experiment without bandage, either a diminished rise in heart rate or a lessened decrease in pulse pressure or both. A graph of a single experiment, figure 4, shows the nature of the effect and table 3 summarizes the results.

The evidence thus suggests that the response without abdominal support is by no means the best response which the individual might make. The widening of pulse pressure with the bandage, often conspicuous, seems to show a greater output per beat from the heart, and taken with the sense of well-being experienced by practically all subjects indicates a better brain supply. An instance of this is a subject in series VI who habitually became dizzy on standing. She was unable to stand more than eight minutes without support but with support stood easily for the whole fifteen-minute period.

These experiments can give no idea about the amount of blood which usually stagnates in the abdominal organs during standing, but they indicate decidedly that such stagnation occurs and that it can be lessened by such a support as the bandage gives. The subjects of posture and abdominal tonus are naturally suggested in this connection (Crampton, 1913, 1915;

Sewall, 1916, 1919; and others). No observations were made in this work which throw light on the possibility of training to better circulatory performance in standing, though such possibility is suggested by the phenomena of convalescence after a long period of illness in bed.

The frequency of vague discomfort, acute dizziness or fainting is of interest. In a total of about 130 experiments on subjects chosen in the

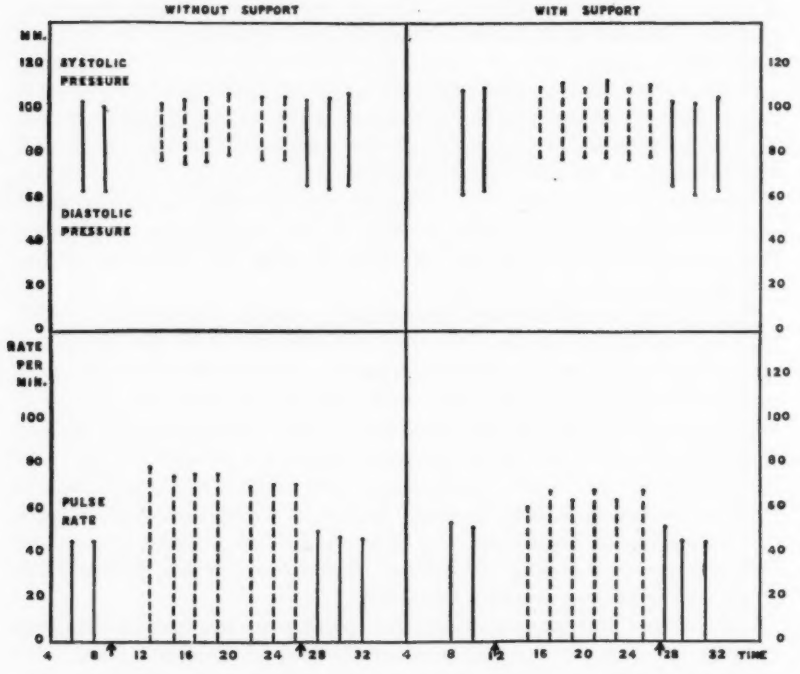

Fig. 4. Findings in typical standing test, with and without support for abdomen, from series VII. Upper chart, pressures; lower chart, pulse rates. Continuous lines, reclining; dotted lines, standing. Abscissa, time in minutes from beginning of experiment. Arrows indicate time of rising and reclining. Note that there is less change in both pulse rate and pulse pressure when abdomen is supported.

main at random there were 10 instances of dizziness or faintness acute enough to stop the experiments and in some cases early in onset so that the findings were too fragmentary to include in averages. There were at least as many more experiments in which the subjects experienced real discomfort though they completed the tests. Some subjects are always uncomfortable in standing, yet these were all young women in "good

health" with no known circulatory defects. The sudden dizziness felt by many persons on rising rapidly usually passes away in about 15 to 30 seconds, a time practically identical with the first adjustment made by the circulatory apparatus for the erect position. This is to be distinguished from the circulatory embarrassment occurring after a prolonged period of standing.

In endeavoring to picture what is happening in these cases of circulatory failure or embarrassment due to gravity it is natural to look to other occa-

TABLE 3

Effect of abdominal support on circulatory findings in prolonged standing. Series VI. Several different supports used; 16 experiments on 16 subjects. Series VII. Broad ACE bandage; 13 experiments on 13 subjects

All figures are averages. In no experiment did the characteristic result fail to appear in either heart rate or pulse pressure or both. Standing averages from minutes 3–15 of standing period. Reclining averages from control period after standing.

	HEART RATES, BEATS PER MINUTE							
	Without support				With support			
	Reclining	Standing	Increase	Per cent increase	Reclining	Standing	Increase	Per cent increase
Series VI	59.5	84.15	24.65	41.4	59.7	78.6	18.9	31.7
Series VII	58.0	87.7	29.5	50.9	57.2	80.6	23.4	40.9
	PULSE PRESSURES, MILLIMETERS Hg							
	Without support				With support			
	Reclining	Standing	Decrease	Per cent decrease	Reclining	Standing	Decrease	Per cent decrease
Series VI	46.9	33.1	13.8	29.4	44.5	35.6	8.9	20.0
Series VII	35.2	25.0	10.2	29.0	37.7	28.2	9.5	25.2

sions where there are similar elements in the picture. If the blood stagnates there will be a relatively small volume of blood circulating and a resulting tendency to low oxygen and inadequate pressures. In at least very many if not all conditions of low oxygen the heart-rate is accelerated whether the occasion be one of increased use of oxygen or of diminished supply. In standing as the metabolism tests have shown there is no reason to consider the increased rate due to increased use of oxygen, but have we not in the evidence for stagnation with its consequent decreased volume of circulating blood abundant justification for explaining the rise of heart

rate as due to low oxygen supply? This would be entirely in harmony with the work of Schneider (Lutz and Schneider, 1919; Schneider, 1921; Schneider and Truesdell, 1921, 1924) who refers to the rise of heart rate in low oxygen as a symptom of distress, not of compensation. Schneider also found two types of response, the fainting and non-fainting types, which approximately parallel the differences between those young women who never get dizzy in standing and whose pressure determinations corroborate their words and that other group who if not acutely dizzy are always more or less uncomfortable when standing and who show especially rapid heart rates and very narrow pulse pressures. In the consideration of high altitudes Barcroft (1925, chapter VIII and appendix I on Mount Everest reports) cites the rise in heart rates in unacclimatized persons and ultimately in all persons as a consequence of low oxygen. He relates the phenomena to anoxemia of the medullary centres and the ultimate slowing of the heart, seen by us at the very dizzy or faint stage, to intense vagal stimulation. We keep in mind, also, the possible importance of a poor coronary circulation due to the low output per beat in an inefficient heart.

The records of the effects of low oxygen in chamber experiments do not, as far as we have found records, show the rise of diastolic pressure and narrow systolic pressure so characteristic of the quiet standing position. A parallel to this may perhaps be found in the effects of hemorrhage reported by Meek and Eyster (1921) where the lessened volume of the blood after considerable hemorrhage in dogs was accompanied by a maintenance of mean and diastolic pressures but by a fall of systolic and pulse pressures. The amount of blood removed from the circulation in these experiments is of the order of magnitude of the lessened return found in standing subjects by Field and Bock (1925) and by Turner (1927a). It is suggested, therefore, that the vaso-constriction which serves to raise the diastolic pressure in standing and which shows signs of failure at the time of subjective discomfort is distinctly as in hemorrhage a compensatory mechanism. As in that case, the lessened output of the heart per minute as well as per beat is an essential element of the picture and due to lessened return of blood from the veins. This output is still adequate for the needs of the brain in the many who stand successfully but it is wont to fail more or less as the quiet erect position is held and in poor subjects the time of successful functioning of the medullary centres may be brief indeed. It is of the greatest importance to note that the methods which normally assist in returning blood to the heart are those which relieve this circulatory embarrassment in standing, deep sighs are frequent in these uncomfortable persons, restless movements are often almost beyond their control, they stand much better during the abundant circulation which follows a meal when return from the digestive tract would be especially facilitated, they are aided by

abdominal supports. As above suggested an interesting question awaits an answer, can the circulatory adjustments in prolonged quiet standing be definitely trained to better performance in the majority of healthy young women?

SUMMARY OF RESULTS

1. By a series of 31 experiments on 12 healthy young women it was determined that the rise of metabolism due to tilting the subject to 62° was practically negligible while circulatory adjustments in heart rate and arterial pressures were almost as great as when the subject stood. The rise in metabolism in quiet standing was 19 per cent, a rise which considered as exercise would by [illegible]ans account for the marked increase in heart rate. A distinct [illegible] the response to gravity as contrasted with exercise is thus suggested, though both are characterized by increased heart rate. Exercise shows a widened pulse pressure, standing a narrowed pulse pressure.

2. A second series of 26 experiments on 12 subjects verified the general circulatory findings of series I but the omission of the movements attending the metabolism determinations revealed a difference in the gravity reaction for 60° and for 90°.

3. The delicacy of the reaction of the circulatory apparatus to gravity for short periods of six minutes was further tested by series III, 17 experiments on 11 subjects, in which the response to tilting, head up, to each of the following angles, 15°, 30°, 45°, 60°, 75°, 90°, was found to be distinct and progressive for both increase of heart rate and narrowing of pulse pressure.

4. Preliminary experiments indicate that whereas the reaction with head up leads to higher heart rate and narrowed pulse pressure, the opposite is true for short periods in the head down position, decreased heart rate and widened pulse pressure.

5. The progressive increase in the volume of the legs to a point above the knees was determined by quiet standing in a can of water for fifteen minutes to average 175 cc. for series IV, 35 experiments on 20 subjects in the winter season, and 256 cc. for series V, a short series of 6 experiments on 4 subjects in summer. Series IV gave an increase of 1.9 per cent in leg volume, series V an increase of 3.36 per cent. This increase in volume may be explained by the stagnation of circulating liquids in the legs during quiet standing.

6. That stagnation of blood occurs in the abdominal region during quiet standing is indicated by series VI, 16 experiments on 16 subjects, and by series VII, 13 experiments on 13 subjects. In each experiment circulatory determinations were made during a fifteen minute period of quiet standing with no abdominal support and for a similar period with the firm support

of corset or bandage. The response was markedly affected by the support, in that the rise of heart rate and the narrowing of pulse pressure were less than in the control experiments. Such changes are explained by the prevention of blood stagnation by the support and the consequent lessened need for adjustment because of better return to the heart than when the abdomen has only the usual muscular support.

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A SPECTROPHOTOMETRIC STUDY OF MUSCLE HEMOGLOBIN

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Received for publication April 17, 1930

Since the first observations on the red pigment of muscle by Boerhaave in 1775, the nature of this substance has been a moot question. Nor have modern methods and an increased knowledge of blood pigments clarified the situation. Two well-defined schools of thought exist to the present time, i.e., those who claim this red pigment to be identical with the hemoglobin of the blood and those who do not.

Mörner (1897), as a result of his spectroscopic observations, was the first to call attention to the fact that the alpha absorption band of muscle pigment did not occur at 575 millimicra as in the case of blood oxyhemoglobin, but at 580. Günther (1921) confirmed this observation and was so impressed by the constancy of the variation that he suggested the name "myoglobin" in place of the more common term, muscle hemoglobin. Opposed to this is the observation of Keilin (1925) who again confirmed the position of the band at 580 millimicra, but calls attention to the fact that the deviation is not much larger than one observes in hemoglobins from various species and, therefore, rightly decries the introduction of a new name.

The most recent observations are those of Kennedy and Whipple (1926). These investigators used the spectrophotometer for their determinations and studied the color of solutions extracted from chopped red muscle by dilute ammonia. They agree with previous observations as to the position of the bands, but suggest that the cause of the deviation from blood hemoglobin is due to fermentation occurring during the process of extraction. They suggest, therefore, that there is no essential difference between muscle and blood hemoglobin. In a later paper, Whipple (1926) shows that muscle hemoglobin is undisturbed over a comparatively long period of anemia, indicating it cannot take the place of blood hemoglobin.

In light of the confusion in regard to this substance it is obvious that further work of a critical nature is needed. We have, therefore, repeated some of the work of other observers and have amplified these observations both as regards methods and detail of observation.

METHODS. Examination of the pigment of muscle was made by means of a Keuffel and Esser spectrophotometer. The mode of observation varied with the type of material used. Extracts of tissue were made according to the method described by Kennedy and Whipple (1926), i.e., by allowing chopped muscle from a thoroughly perfused animal to stand for 24 hours in a solution of ammonium hydroxide. The solution was then carefully filtered and the absorption curve determined. This method is not above criticism. Kennedy and Whipple themselves realized the possibility of fermentation occurring as a result of the action of tissue juices. One must also account for the action of extractives other than hemoglobin influencing the absorption bands. Kennedy and Whipple attempted to compensate for this by using puppy muscle which is nearly colorless. There is no positive proof however that the use of immature muscles will compensate for the effects of the materials which may be extracted from mature muscles where we know soluble substances to be far more concentrated.

These drawbacks led us to adopt an entirely different method by which

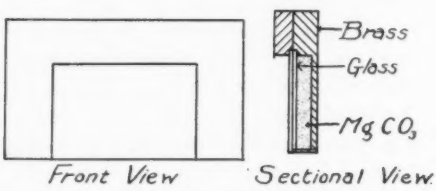

Fig. 1. A diagram of the cell used for obtaining reflection curves of tissue. The two plates are compressed by thumb screws.

the muscle pigment could be studied *in situ*. This method is based on determinations of the reflection curves from uninjured muscle immediately after its removal from the perfused animal. In this manner the effect of chopping and extracting for a long period is obviated. By comparing the reflection curve thus obtained with the curve found in the case of extracts we should be able to determine the effect of contaminating substances upon the absorption curve, for in the muscle we have a high concentration of such material, while in the extract a low concentration. Thus, any effect of the physical characteristics of the solute, such as refraction, should produce a definite variation between the observation of the extract and that of the muscle.

The manner of making reflection measurements was very simple. The Keuffel and Esser spectrophotometer uses two magnesium carbonate blocks as reflecting surfaces. We replaced the upper block with a cell such as that diagrammed in figure 1. By means of such a device we were able to press out the muscle to a thin layer from which we consistently

obtained definite and characteristic curves. We compensated for the effect of the glass of the cell by the introduction of a similar thickness of the same glass on the lower reflecting surface.

All tissues examined were taken from animals which had been perfused until the perfusing fluid (0.9 per cent NaCl) was practically colorless. In those experiments in which rat tissue was studied the procedure was to open the thorax of the animal and snip off the tip of the ventricles. A cannula was then inserted into the aorta and about 2 liters of saline forced through the vessels. The success of this type of perfusion will be shown in the experimental results.

Two methods were used in the case of dogs. One, that described by Kennedy and Whipple, consisted of alternate bleeding from the carotid and injection of saline into the jugular. The other method was to insert cannulas into the dorsal aorta and vena cava and thus perfuse the hind quarters. Both methods gave excellent results.

EXPERIMENTAL RESULTS. It is obvious that the first and most desirable point for attacking the problem is to determine the absorption curve of muscle oxyhemoglobin, both in extract and *in situ*, and to compare the curves thus obtained with oxyhemoglobin obtained from the blood. This was done and the results of one of the several experiments are shown in figure 2. Curve *A* is that of oxyhemoglobin of the dog's blood. It is characteristic and its constants, when calculated, are those which have been established for oxygemoglobin. Curve *B* is the absorption curve of an extract of the perfused thigh muscles of the dog made according to the method of Kennedy and Whipple. The curve is exactly the same as those obtained by these authors. In comparison with curve *A* every characteristic peak or dip of the curve is moved 5 millimicra toward the red end of the spectrum. Curve *C* offers an interesting comparison. It is the reflection curve of a piece of perfused diaphragm. The observations were started within 10 minutes following the cessation of perfusion. Since the muscle was tightly pressed in the cell it was found that unless some means of oxygenation was adopted the curve of reduced hemoglobin was obtained. Therefore the technic of soaking the muscle in dilute hydrogen peroxide for a brief period preceding the observation was adopted. In some cases potassium cyanide was added to the peroxide solution but it was found that this was not essential.

With the exception that the peaks are not as high as in the case of the transmission curves—due to the low reflectivity of the muscle—we find it identical with the curve of the extract. It must be clear then that the distortion of the muscle hemoglobin absorption curve cannot be due to fermentation. Certainly the period between the removal from the dog and the reading is far too brief to permit any extensive change. We can say definitely that the spectral characteristics of muscle hemoglobin *in situ* are the same as those of the extract.

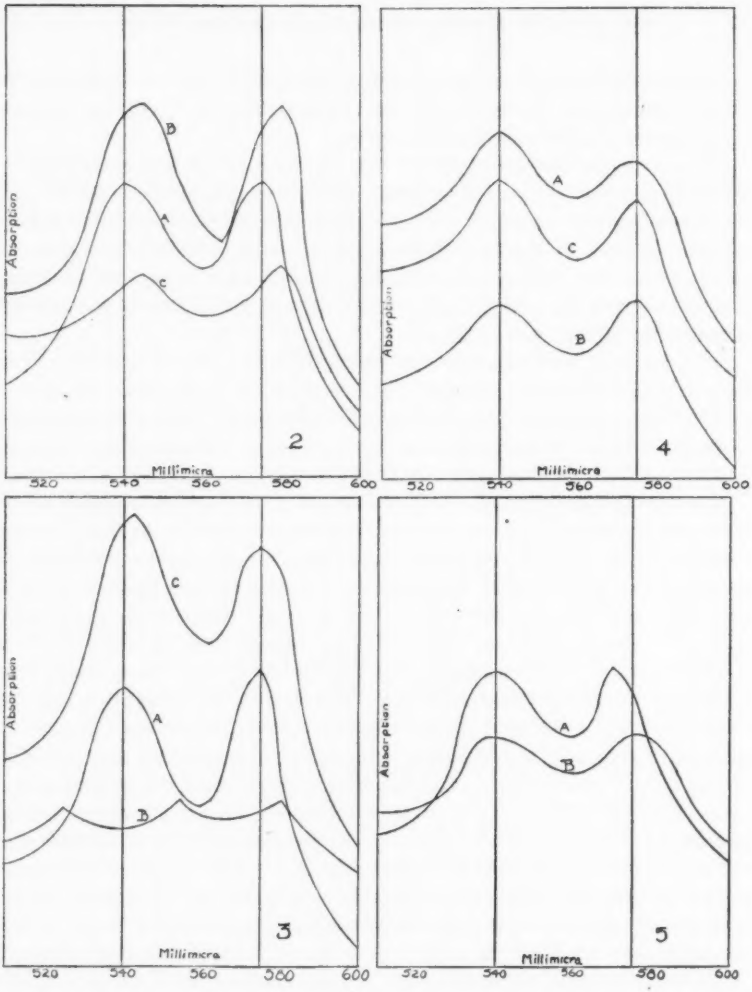

Fig. 2. A comparison of the absorption curve of oxyhemoglobin, *A*, with that of the pigment extracted from chopped muscle, *B*, and that obtained from the reflection of fresh muscle, *C*.

Fig. 3. The effect of absorption by the basic color of muscle, *B*, on the absorption curve of oxyhemoglobin, *A*. Curve *C* is the result of reading *A* and *B* at the same time.

Fig. 4. The effect of the presence of various decomposition products of hemoglobin on the absorption curve of oxyhemoglobin. Concentration of each product approximately 50 per cent of the total pigment. *A* is the effect of methemoglobin; *B*, the effect of hematin; and *C*, hematoporphyrin.

Fig. 5. A comparison of carboxyhemoglobin from blood and muscle, illustrating the variation in the span.

A second question to be considered, the answer of which is found in a comparison of the reflection curve and the extraction curve, is the physical effect of solutes other than pigments. It will be noted that the distortion of the muscle hemoglobin curve is characterized by a complete shift toward the red end of the spectrum. Kundt formulated the rule that the presence of substances which cause an increase in the refractive index of the solution caused a shifting of the absorption bands towards the red end. This "rule" has been shown (Baly, p. 468) to hold in about 50 per cent of the cases examined. Even with this discrepancy it is interesting to consider in connection with the above mentioned distortion, since here we find a shift towards the longer waves.

The refractive index of a solution is proportional to the concentration of active material. Hence, we should expect a more marked shift in the case of the reflection measurement than in the case of the extract where the concentration of optically active substances must be low. We have measured the refractive index of our extracts and find them to be but very slightly higher than that of the 0.4 per cent ammonium hydroxide used for dilution. Since the curves agree we may dismiss this factor as causing the observed difference.

There is another factor that may modify the absorption curves of oxyhemoglobin which must be considered. Keilin (1925) called attention to a pigment, cytochrome, of well-nigh general distribution. It has been found in both plant and animal tissue. The question arises whether or not the presence of this fundamental pigment may not cause the observed shift by producing an absorption curve which represents a fusion of the absorption curve of blood hemoglobin with that of cytochrome.

While this possibility is worth testing one point must be kept in mind. Keilin has found that it is only in the reduced stage that cytochrome shows absorption bands. One would, therefore, hardly expect it to have an influence on the color of well-oxygenated muscle pigment. Nevertheless we have made studies of the basic color of washed white muscle and its effect on the absorption curve of hemoglobin.

Figure 3 represents the result of one of these experiments. The animal used in these experiments was the white rat for we found that thorough perfusion of this animal gave us white muscle. We would have preferred to use the dog, but unfortunately the dog has no white skeletal muscle. This observation is contrary to certain European observers who claim to have found the psoas muscle without pigment. We have dissected out the psoas group from 5 dogs and in every case the muscles were red.

Curve *A* is the absorption curve of blood oxyhemoglobin from the rat. Curve *B* is a curve obtained by plotting the negative log of the light reflected from the abdominal wall muscle of the same animal. No trace of the presence of hemoglobin is present. Nevertheless we see a definite

absorption curve with three small but well-marked peaks. The curve is extremely interesting in that we have found identical curves from washed frog muscle and alkaline acetone yeast. It has, one will note, an absorption band at 520 millimicra, one at 555 millimicra, and a third at 580 millimicra. The first two correspond with the bands of cytochrome as found by Keilin. We have not found any mention of the band at 580 millimicra, in spite of the fact that we have seen it consistently. It will be recalled that the technic of making reflection measurements prevented the access of oxygen. This tends to make cytochrome visible, since the bands are only seen in the reduced state. If this colored compound is cytochrome the curves have the additional interest in that they are the first published absorption curves of this compound.

Curve *C* is of particular interest. It represents the result of reading the transmission of the rat's blood simultaneously with the reflection of the muscle; in other words, a fusion of the curves given as *A* and *B*. It is the type of curve one should obtain for muscle hemoglobin if the distortion were due to a second pigment when this second pigment is the basic color of muscle, i.e., what we have suggested may be cytochrome. A shift towards the red end occurs at the *beta* band (540 millimicra), but we do not find a marked shift in the band at 575 millimicra. There is, therefore, no evidence that cytochrome can distort the curve of hemoglobin in such a manner as to produce the characteristic curve shown in figure 2.

One must not, however, disregard the possibility that hemoglobin derivatives may be the cause of the distorting influence. Kennedy and Whipple suggest that these may be the factor causing the variation between the two types of curves they present. Our own experience with the effect of hemoglobin derivatives on the absorption curve of oxyhemoglobin leads us to doubt this possibility. Since, however, most of the published absorption curves are those of pure compounds it is extremely desirable that we present curves demonstrating the effect of certain products of hemoglobin decomposition on the absorption curve of oxyhemoglobin.

The first step which one may conceive as taking place in the breakdown of hemoglobin is the simple oxidation of the iron from the ferrous to the ferric state, i.e., hemoglobin to methemoglobin. Curve *A* of figure 4 shows the distorting effect of converting about 50 per cent of the hemoglobin to methemoglobin. There is a definite change in the absorption curve from that found in the case of oxyhemoglobin, but it is not that found in the case of muscle hemoglobin.

Suppose that a further change has taken place in the hemoglobin and the methemoglobin formed has been split into hematin and the protein base. Curve *B* of figure 4 shows the change occurring in the absorption curve when one-half the pigment is destroyed in this manner. The curve thus found does not resemble that found for muscle pigment any more than that of pure oxyhemoglobin.

Finally, let us suppose that decomposition has proceeded to the point where one-half the blood has been broken down to the point where the iron has been split off from the hematin, i.e., to hematoporphyrin. The resulting absorption curve is given as curve *C* of figure 4. As in the preceding curves the maxima occur at 540 and 575 millimicra, as in the case of oxyhemoglobin from the blood.

Obviously then if the distortion is due to fermentation products decomposition has proceeded beyond this point. This we do not believe is the case. We have also accounted for hemochromogen if our basic absorption curve of muscle color is that of cytochrome, since Keilin has shown cytochrome to be a mixture of hemochromogens.

Figure 5 presents the most definite and interesting proof of the identity of muscle hemoglobin. We show in these curves a comparison of carboxyhemoglobin from blood (curve *A*) and muscle (curve *B*). It will be seen that curve *B* shows an *alpha* band distinctly to the right of curve *A*. The interest in these curves is not however aroused by this fact but by the variation between the positions of the *alpha* band of carbon monoxide hemoglobin in each case from the corresponding oxyhemoglobin. Anson, Barcroft, Mirsky and Oinuma (1925) have shown that the change in position of *alpha* band occurring as a result of carboxyhemoglobin formation varies with the species of animal selected as a source of hemoglobin. Thus, in man the shift (or span, as Barcroft designates it) is 60 A.U.; in the dog it is 58 A.U.; while in the rabbit it is only 40 A.U.

Let us apply this same test to muscle hemoglobin. We find that the peak of the *alpha* band of carboxyhemoglobin from this source occurs at approximately 577 millimicra or 30 A.U. from that of oxyhemoglobin from muscle. This "span" is far less than any recorded by Anson *et al.* There can be no question that the shift is less than that observed in the dog's blood hemoglobin. Its significance is also interesting, since Barcroft has shown that the span in Angström units is directly proportional to the stability of oxyhemoglobin. Measurement of Kennedy and Whipple's curves shows the same phenomenon.

SUMMARY

To summarize the above data we may make the following statements: Muscle hemoglobin is distinct from blood hemoglobin in that the absorption curves of reduced and oxyhemoglobin both show a characteristic shift toward the red end of the spectrum. This shift is not due to fermentation, as thought by Kennedy and Whipple, since we have found it to occur *in situ* in fresh muscle. These same experiments, as we have pointed out, also show that Kundt's rule does not hold in this case, for if it did the comparatively high concentration of optically active substances in the muscle

should cause a more marked shifting of the spectrum towards the red end, as compared to the effect of the dilute extract. This we have shown is not the case, since the *alpha* and *beta* bands occur at the same wave length in both extract and *in situ*.

Finally, the shift in the position of the *alpha* band occurring as a result of carboxyhemoglobin formation is not the shift one would predict if the pigments were identical with blood hemoglobin. Instead of a 6 millimicra shift we find but 3 millimicra.

It seems, therefore, that nothing we have been able to do has produced a distortion of blood hemoglobin curves in such a manner as to make them comparable with muscle hemoglobin. In light of the evidence we cannot agree with the statement that blood and muscle hemoglobin are identical, but feel that the above evidence points definitely toward a different form of hemoglobin. Without doubt the pigment fraction of both hemoglobins is the same, but as in the case of species variation we have to deal with a different protein fraction as suggested by Barcroft.

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MECHANICAL INTERFERENCE WITH THE CIRCULATION AS A FACTOR IN TRAUMATIC SHOCK

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Received for publication April 19, 1930

Much emphasis has been placed by certain investigators on the importance of interference with the circulation as an etiological factor in the production of traumatic shock. Extensive obstruction of arterial flow or venous return was employed to produce shock in the experiments of Janeway (1914), Janeway and Jackson (1915), and of Erlanger *et al* (1919). In an attempt to determine the effect of interference with the circulation *per se* we have produced this condition by humane methods without the complicating factors of ether anesthesia and operative trauma present in the experiments of others.

Employing ether anesthesia and aseptic precautions, a preliminary operation was performed. Strong but flexible ligatures were placed about the blood vessels to be occluded and the ends of the ligatures brought outside the animal's body. Arrangements were thus made for compressing the thoracic or abdominal aorta against the spinal column or compressing the thoracic vena cava between two interlocking U-shaped ligatures. This procedure occasioned the animal no discomfort. The actual experiment was performed two to seven days after the preliminary operation.

The abdominal aorta was occluded until the femoral pulse became imperceptible or very weak. In four experiments this was maintained for 15, 85, 115 and 127 minutes. No indication of shock occurred in these animals during or subsequent to the period of occlusion.

Obstruction of the thoracic aorta for 165 minutes with a single ligature or with two ligatures for 40, 45, or 110 minutes did not produce the condition of shock. The latter three animals did show the usual symptoms of spinal anemia. This experiment was repeated on an animal lightly anesthetized with ether. At the end of 15 minutes the experiment was terminated because of the development of a marked respiratory disturbance. Recovery of this animal was complete and uneventful.

Occlusion of the thoracic vena cava was maintained until respiration ceased. This occurred after 23, 45, or 53 minutes. Release of the ligatures, combined with artificial respiration, led to a prompt and complete

recovery. No indications of shock were observed. When the same experiment was repeated with animals lightly anesthetized with ether, respiration ceased in 8, 11, or 15 minutes. Only one animal could be revived but its recovery was entirely similar to an animal recovering from simple ether anesthesia.

We may quote the criteria emphasized by Meltzer (1908) and Mann (1914) as indicative of traumatic shock: loss of sensibility, pallor of the mucous membranes, a small weak pulse, irregular, rapid, shallow respiration, markedly lowered blood pressure, subnormal body temperature and depressed reflexes. We wish to emphasize the fact that these symptoms have not been observed in our animals.

Various investigators have considered the following factors of importance in the etiology of shock: interference with venous return, slowing of the blood flow, clumping of the corpuscles, injury or stretching of the capillaries and venules, relaxation of tissues supporting these structures, excessive loss of plasma from the capillaries, tissue asphyxia, formation of histamine-like bodies, bombardment of the central nervous system by afferent impulses arising from the site of the trauma, or acapnia from the respiratory stimulation produced by the same mechanism. In the experiments just described, opportunity was offered for some or frequently all of these factors to become active. Since our investigation has demonstrated that a marked degree of arterial or venous obstruction can be maintained for extended periods without the development of traumatic shock, it tends to throw doubt on the etiological importance of the above mentioned factors.

It appears most likely that our results differ from those reported by other investigators because of the absence in our experiments of two complicating factors: a, ether anesthesia; b, manipulation of the abdominal or thoracic viscera. Our results therefore illustrate in a striking manner the harmful effect of ether anesthesia during a period of interference with the circulation. They also appear to emphasize the injurious nature of manipulation of the contents of the abdominal or thoracic cavities under such conditions.

We may conclude that in the absence of anesthesia or direct trauma, moderately extensive interference with the circulation (arterial or venous) of large portions of the body does not produce traumatic or surgical shock either during or subsequent to such circulatory obstruction.

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CONDUCTIVITY OF BLOOD DURING COAGULATION

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Received for publication April 25, 1930

In discussing the structure of gels, the identity of conductivity of sol and gel has been pointed out, especially by McBain (1), as particularly significant. The absence of any appreciable change of resistance during the tremendous viscosity changes that accompany gelation, is a strong indication that the gel structure is micellar, and that the ionic movement is not hindered appreciably through the interstices of the micellar meshwork. It has been shown that in soap (2) and in gelatin (3) there is no significant change in conductivity upon gelation. It has also been pointed out by Gelfan (4) that the independence between the conductivity of protoplasm and its viscosity, as well as its hydrophilic properties indicates that protoplasm has a micellar structure. A micellar structure of the normal blood clot, in which the presence of a meshwork of needles has definitely been demonstrated, is indicated by the work of Howell (5). Since viscosity increases enormously during the coagulation of blood, a measurement of the electrical conductivity during coagulation would indicate whether the blood clot is similar in its colloidal structure to such gels as soaps and gelatin.

Kugelmass (6) has reported that addition of thrombin to oxalated plasma leads to a sharp rise in resistance which continues up to the point of coagulation. The increase of resistance he interpreted as due, in great part, to a condensation of ions on the surface of the fibrin.

We have employed the Kohlrausch method to measure the conductivity of blood during the process of coagulation. The conductivity cell consisted of a horizontal glass tube with four vertical tubes rising from it. One of the end tubes was used to determine the time of coagulation by a method essentially the same as that of Cannon and Mendenhall (7). The second and fourth vertical tubes contained the platinum black electrodes, and the third tube was used to introduce the blood and during each determination contained a Beckmann thermometer which dipped into the blood.

To obtain oxalated plasma, enough crystalline sodium oxalate was added to the syringe to make a 0.2 per cent solution. The blood after

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having been drawn was shaken thoroughly, centrifuged, and different amounts of calcium chloride were added to the plasma to vary the rate of coagulation. The conductivity determinations were made at room temperature. To eliminate a possible source of error, we did not add any thrombin. As the curves in figure 1 indicate, the conductivity was not influenced significantly by the process of coagulation. The greatest increase in resistance observed was less than 4 per cent. It may also be pointed out that this slight increase in resistance does not seem to depend upon coagulation. Figure 1 shows coagulation to occur before, during, and after the slight rise in resistance. We are unable to account for the divergent results of Kugelmass (6).

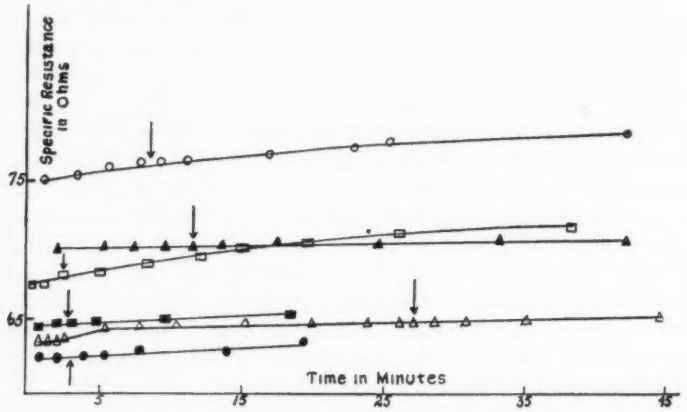

Fig. 1. Blood plasma (dog). Specific resistance plotted against time interval after the addition of CaCl to the oxalated plasma. Arrows indicate time of clotting. Temperatures, starting from the curve having the highest initial specific resistance, in descending order, are 21, 25, 21, 28, 30, 29°C.

In the experiments on normal whole blood, the blood was collected in a syringe heated to body temperature and was immediately transferred to the conductivity cell supported in a constant temperature bath at 38°C. The temperature remained practically constant during the experiment and never varied more than one-half of a degree centigrade. In each of the twelve recorded experiments on whole blood, the first conductivity reading was made within one minute of the bleeding and we were able to make several determinations before coagulation occurred.

In figure 2 the results of four typical experiments on normal whole blood are given. Specific resistance is plotted against the time interval after drawing of the blood. The time of clotting is indicated in each experiment by an arrow. We did not observe in any experiment an increase in resistance during the process of coagulation. In fact, immediately after

coagulation was observed to have taken place, there was in most cases a very slight and temporary decrease in resistance, though this is perhaps of little significance. A sharp rise in resistance did occur about fifteen minutes after the drawing of the blood, or about ten minutes after coagulation. It is important here to distinguish between the absence of any appreciable change of conductivity during the course of coagulation, and the decrease of conductivity that was observed some time after the clot has formed. In the former, the conductivity was independent of viscosity, since the tremendous increase of viscosity that occurs upon coagulation had not influenced the specific resistance of the system. The increase of resist-

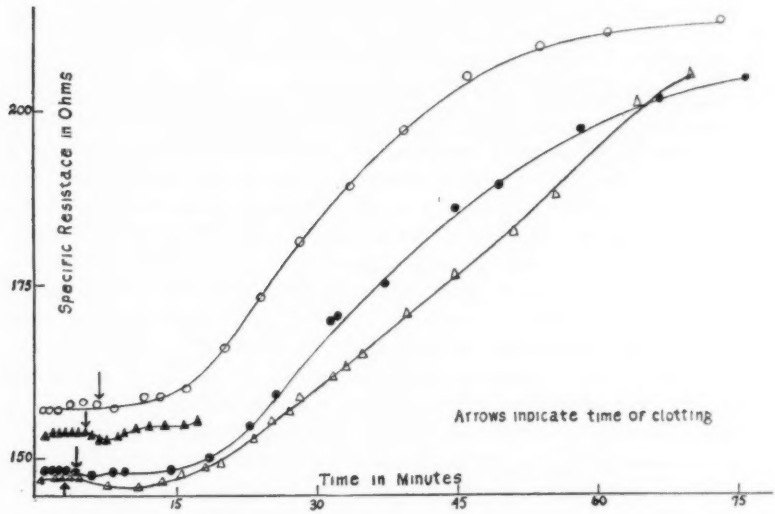

Fig. 2. Normal whole blood (dog). Described in text.

ance that occurred about ten minutes after coagulation was concomitant with syneresis, and depended upon the presence of the blood corpuscles. In plasma this rise in resistance did not take place. The presence of the non-conducting corpuscles in the dense fibrin mesh-work, when the clot contracts, apparently reduces the mobility of the ions sufficiently to increase the resistance.[2]

[2] During the preparation of this manuscript our attention was called to a note by R. T. Frank, 1905, This Journal, xiv, 466, in which he gives a purely qualitative report on the same experiment. Having undertaken this experiment with the purpose of using the conductivity change during coagulation as a means for other studies, this author reports negative results. The experiments were apparently not carried beyond the point of coagulation since the rise in resistance that we find about ten minutes after coagulation is not mentioned.

We feel justified in concluding that the conductivity of whole blood or blood plasma is not influenced by the process of coagulation and is consequently independent of the viscosity changes occurring at this time. The increase in the resistance of the whole blood after it has already coagulated is apparently due to the presence of the corpuscles, for the conductivity of plasma is practically the same during and after coagulation. Since conductivity studies indicate that the fibrin gel is similar to such reversible gels as gelatin, soap, agar, and silicic acid, additional evidence is offered in support of the micellar structure of coagulated blood.

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STUDIES ON THE PHYSIOLOGY OF REPRODUCTION IN BIRDS

XXVII. The Age Distribution of Mortality in Bird Embryos and Its Probable Significance

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Received for publication April 26, 1930

It is an odd fact that, although the bird embryo has long been an object of special biological interest as well as of much commercial importance, only a single study has hitherto been made of the age distribution of mortality throughout the period of embryonic life. It is equally notable that even that lone study concerns only a single species—the common fowl. Since the time of Payne's (1919) study of mortality in chick embryos a few workers have noted that their data for one or another part of the embryonic period agree well with those of Payne; also, some special studies have been made on the age distribution of the death of certain types of abnormal fowl embryos. Payne observed that specially high mortality rates cluster about the fourth and nineteenth days of incubation (fowl). But no one has sought to learn whether these peaks of high mortality were of general applicability and significance in the bird world or whether they applied only and peculiarly to the domestic fowl. The causes of the two periods of high death rate have been very little considered, and hitherto never considered at all as a phenomenon common to bird embryos generally. Dunn (1923) has, however, discussed the whole question of hatchability in the fowl from many other points of view, and has stated our present knowledge so ably that his paper should be read in connection with any special contribution to the subject.

In this paper we supply data for the distribution of embryo mortality in three additional bird species, and for the first time the question of embryo mortality can now be examined on the basis of data which probably apply to birds in general. Our three additional species all belong to the order Columbae, and to two different families of this order. It is found that though the incubation periods of these doves and pigeons differ from each other, and from that of the fowl, nevertheless *the embryos of all of these four species show two distinct periods of high mortality;* and, again, that these two peaks of mortality *occur at essentially equivalent points of the incubation*

period in all of the four different groups. This is the definite and positive result to be reported here. Two periods of high death-rate—the first occurring very early in development and the second very near the end of incubation—may now be regarded as phenomena probably associated with the general method of avian reproduction.

We believe that our long and varied studies on the embryos of doves and pigeons contribute to an explanation of the high death-rate at these two special periods. This, however, is a very complicated problem, and the suggestions which we now venture—because of the real importance of the matter—are made mainly for the purpose of directing attention to the kinds of investigation which must later provide a definite solution. This aspect of the problem, however, must be treated as briefly as possible in this paper. We shall refer to one series of facts which suggest that a part of the abnormally high mortality at the 2- to 4-day stage results, in the broadest sense, from failures in respiratory adjustment; and to another series of facts which indicate that a notable part of the very high death-rate which occurs just before the end of incubation is ascribable to an inadequate water supply at this special period. If these suggestions are well founded it follows that the very method or plan of avian reproduction (egg-building) frequently imposes conditions to which some normal embryos can not become adapted. Though a part—and only a part—of the defective embryos are eliminated by death, this elimination also includes many of the good and fit. Indeed, it is highly probable that a fair fraction of the abnormals and monsters which are eliminated in the second and final period of high mortality were themselves *produced* (in the first special period of hazard) by temporarily successful responses of embryos to meet the unusual or difficult conditions of their gaseous environment. Thus the problem of abnormalities and monsters also enters into this consideration of the causes of prenatal death.

DATA ON EMBRYONIC MORTALITY IN DOVES AND PIGEONS. For this study we have used only a fraction of the data actually available to us. The ages of dead embryos have been recorded continuously in our material during a period of 19 years. We here use only data from those years, or periods, during which we obtained accurate and adequate information concerning the external and accidental causes of the prenatal death; practically all embryos dead from such causes could be and have been excluded from the tabulated results. To all of the data obtained between 1912 and 1917 are added other data for the year 1921–22 (ring doves), and the year 1926–27 (common pigeons). During the two last-named periods measurements were made of the adequacy or thickness of egg shells, i.e., of shells containing hatchable young and of shells containing dead unhatched embryos.

The age distribution of 2010 dead embryos belonging to three groups of

doves and pigeons is given in table 1. There are 1092 embryos of ring doves (*Streptopelia risoria*, and its hybrids with *St. alba*). From other species of wild Peristeridae (*Turtur*, *Spilopelia* and *Zenaidura* sp.) there are 229 embryos. The incubation period in these latter species is slightly less than 14 days. In the ring doves this period slightly exceeds 14 days. From common pigeons (*Columba*, various domestic strains or varieties) 789 dead embryos were obtained. The length of the incubation period here is 17 to 17½ days. It was possible to make a more accurate classification of embryonic age in the youngest embryos (aged 1 to 3 days) than for any later stages; the later stages are therefore grouped in 2-day periods. This method of classification was also partly required by the circumstance that as many as five different persons took part in designating the age of these embryos.

TABLE 1

Age distribution of dead embryos in doves and pigeons

KIND OF BIRD	NUMBER OF EMBRYOS DEAD AT VARIOUS AGES (IN DAYS)								
	1	2	3 and 4	5 and 6	7 and 8	9 and 10	11 and 12	13 and 14	Total
Ring doves	100	132	267	116	79	55	67	276	1092
Wild doves	22	32	47	23	11	11	18	65	229

	NUMBER OF EMBRYOS DEAD AT VARIOUS AGES										
	1	2	3	4 and 5	6 and 7	8 and 9	10 and 11	12 and 13	14 and 15	16 and 17	Total
Common pigeons	50	99	110	92	55	29	20	14	46	174	689

It will be observed that the mortality rate is relatively high in the 1, 2, 3 and 4-day embryos in the case of all of the three kinds of birds. Also that the point of maximum mortality within this 4-day period is reached either on the second or the third day in all of the three groups. Only in the case of the "wild doves" (Peristeridae) is it not entirely evident that the highest mortality rate occurs during the third day; there the total numbers involved are fewest, the second day is possibly the high point, and this is the group having the shortest incubation period. It will next be observed that in all of the three groups a second period of very high mortality occurs during the last two days of the incubation period. This high point occurs during the final two days, irrespective of the total length of the incubation periods (which vary from slightly less than 14 days to slightly more than 17 days). It thus becomes clear that almost the first

days, and again the very last two days of embryonic life are periods of specially high mortality for embryos belonging to three different genera and to two different families of birds.

Payne obtained the age distribution of 2142 dead embryos of the common fowl. For the purposes of the present paper, and for still other reasons, it seems necessary to construct polygons of distribution from Payne's relatively inaccessible data, and to construct similar polygons for our own

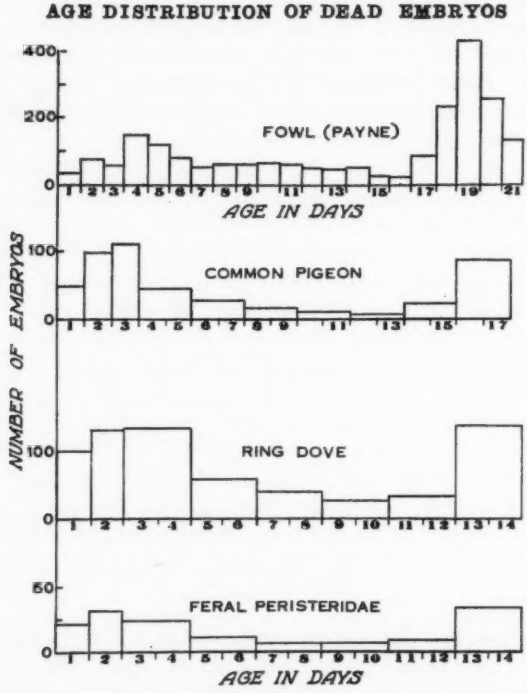

Fig. 1. Distribution polygons showing two periods of high prenatal mortality (near the beginning and near the end of incubation) in embryos from four kinds of birds.

three groups of data, *figuring the four groups on a directly comparable scale.* This has been done in figure 1. Viewing the polygons for the four groups of birds it will be found that a straight and nearly vertical line can be laid over, or connect, the central point of the early period (3–4 days) of high mortality in them all. A similar straight and quite vertical line will connect the highest points of the second period of high mortality in the four polygons. It thus appears possible to conclude that these two points of

high mortality are probably characteristic of bird embryos generally. In other words, periods particularly difficult or hazardous for bird embryos are encountered at two points—a first period placed near the beginning, and a second period practically at the end, of embryonic life.

THE CAUSES OF SPECIALLY HIGH MORTALITY AT EACH OF THE TWO PERIODS. *General statement.* In all probability prenatal death in birds results from several general and specific causes, and many of these causes presumably bring death at any and all stages of development. Certainly some of these deaths are traceable more directly to external factors of incubation, and others more directly to the genetic composition of the embryos. Still other deaths, the writer believes, are traceable more directly to very wide variability in egg structure and composition. This involves the view that, though wonderful to the point of being frequently successful, the avian egg is often an incompletely adapted vehicle of reproduction. The method of avian reproduction requires that water be stored in such quantity as will still be adequate at the *end* of incubation; but the very means (thick shells) which the egg uses to restrict the rate of water loss may, if carried too far, adversely restrict the particular rate of gaseous exchange which is most favorable for early developmental stages. Imperfect adjustments of this type tend to increase mortality at two points—near the *beginning* and near the *end* of embryonic life.

Sub-optimal incubation conditions may result in death at all embryonic ages, and in this paper this aspect of the subject will not be discussed. The rôle of genetic endowment does require immediate consideration. In a case of complete or nearly complete linkage between a factor for recessive white plumage and a recessive factor which caused the death of fowl embryos pure for it, Dunn (1923) showed that the embryos died before the sixteenth day of incubation. There seems to be no evidence that such embryos die in unusual numbers during the third and fourth days of incubation and they are quite absent from the final period of high mortality. Dunn and Landauer (1926) found that homozygous "creeper" fowls die at the 4-day stage; but this gene is apparently not widely distributed among ordinary fowls, and Landauer notes that Payne's material was probably free from it. The chondrodystrophic embryos of Dunn (1923, 1927), Landauer and Dunn (1926) and Hutt and Greenwood (1929a) all die—mostly before the 19-day stage, but apparently at all stages. Their number (perhaps 1 to 10 per cent of all embryos) varies greatly with region and season. Though the mother is certainly involved in the appearance of this disease in her progeny, Dunn's latest study makes it rather probable that the disorder is not of truly genetic origin. Of course it may be expected that other lethal factors will later be recognized; whether their combined action will be found to result in excessive mortality near the beginning and near the end of incubation is a matter now quite unknown.

Monsters and gross structural abnormalities account for only a relatively small percentage of deaths in either fowls or pigeons. Among about 3,000 fowl embryos dead at 17 to 22 days Dunn found only about 5 per cent of deaths from these causes; Hutt and Greenwood (1929b) found "at least 3.6 per cent" among nearly 12,000 dead germs. Such deaths therefore account for very little of the 48.7 per cent of the total mortality which Payne found to occur at 18–21 days of incubation. In both doves and pigeons I have found abnormal embryos dead at all ages, though probably more of them die during the last two days of incubation than during any other similar time interval. In pigeons, as in fowls, the total number of such embryos is too small to have a very marked influence on the distribution of the total mortality. Some monstrous developments arise from genetic causes while still others—probably a much greater number—are certainly induced by the conditions of incubation, or by an interplay of these conditions upon variable egg structure. This same statement probably applies also to the malpositions considered in the next paragraph.

Of considerable importance are deaths due to malposition of the embryo, since these deaths are rather numerous and distinctly tend to occur in greater number at or near the end of incubation. Sanctuary (1925) found that 13.15 per cent of "full-time" dead chick embryos had the head buried between the thighs; Hutt (1929) found the same in 9.25 per cent. It is fairly evident that such embryos can not hatch. Moreover, it is probable that still other abnormal positions of the head sometimes prevent hatching and result in death at or near the hatching period. Hutt found that 56 per cent of all of the embryos dead at 18–22 days were in one or another of four types of malposition. Clearly, however, the death of only a part of these embryos is attributable to malposition, and perhaps the death of few or none of the 18 and 19 day embryos of his tabulation can properly be traced to malposition. It thus appears that all known causes of embryonic death at the 18–22 day period fall far short of accounting for the 48 per cent of total deaths which Payne and others have found to fall within this period. Similarly, the excessive mortality of the very early days of incubation remain very incompletely accounted for.

Respiratory adjustments and death of young embryos. Much older work has shown that marked changes in the oxygen supply of the very young embryo of the fowl may cause its very early death. In some older work, and in the more recent work of Stockard (1914), it was noted that early stages of the fowl embryo are also markedly sensitive to the gases and fumes sometimes present in laboratory air. Weldon (1902) reported that by keeping the air cavity of the egg filled with water during the early days of incubation he frequently succeeded in preventing the formation of the amnion. Several workers have shown that a reduction of the oxygen supply to young chick embryos also leads to the production of monsters;

and one rather unsatisfactory earlier report stated that high pressures of oxygen also produce abnormalities and monsters. Aggazzotti (1913) found that embryos in eggs taken to high altitudes for incubation suffer from anoxemia and show abnormally high death rates (see also Wesselkin, 1913). During recent years this laboratory has studied, extensively and quantitatively, the effects of altered pressures of oxygen on mortality and on monster-production, in variously aged embryos of both doves and pigeons (1921b, 1923b). A few similar tests were made on fowl embryos (1924). We now conclude—from the facts thus obtained and previously published by us and from the results of the investigations of others on the fowl embryo—that the early period of high mortality in embryos of all these birds is, to a considerable extent, the result of relatively numerous failures of respiratory adjustment at this period. The ordinary gaseous environment of the avian *egg* is a notably uniform and constant thing; but, because of variable egg and shell structure and the numerous variable conditions (temperature, etc.) which influence the gaseous intake and requirements of the changing embryo, the gaseous environment of the avian *embryo* is one of variable and changing hazards—and one to which fair numbers of quite normal embryos can not become adapted.

In a further brief summary of the evidence (particularly that from our own studies) bearing upon the above conclusions it is expected that the publications cited will be consulted for a closer view of the evidence, for the earlier literature, and for much related material. In a paper dealing with the asphyxiation of embryos in eggs retained abnormally in the oviduct (Riddle, 1923) the following facts were established: *a*. Among pigeons and doves about one egg in every 100 produced is retained in the oviduct during an excess or abnormal period which varies from 5 hours to many days. In all these cases the earliest development, which normally occurs with the egg exposed to air, must occur in the more restricted oxygen supply (and a higher temperature) prevalent in the oviduct or uterus. *b*. About one-half of the embryos in eggs thus retained are killed (usually at the 0.5-day stage) as a result of this retention. *c*. Death always occurs within *thick-shelled eggs* which are retained for the longer periods. It was thus made highly probable that the death of these particular embryos results from a limitation of the oxygen supply.

Other studies demonstrated *a*, that during the first three days of development dove and pigeon embryos are far more sensitive to increased pressures of oxygen and far less sensitive to diminished oxygen supply than are any other embryonic stages (for curves, see 1923b). But the *second* day (near the end of which the formation of hemoglobin begins) occupies a very special position within this 3-day period as well as in the whole period of embryonic development. Embryos of both doves and pigeons, when taken during the second day (1.0 to 1.9 days) and subjected during the next 24

hours of their development to altered pressures of oxygen (1791 tests; 1923b), were *least* often killed by a diminished oxygen supply and most often killed by an increased oxygen supply. "Age therefore becomes the chief factor involved in the death or survival of embryos subjected to altered oxygen pressures" (1921b). *b.* The first day stage (0.0 to 0.9), however, was shown to be the stage at which abnormalities and monsters are most readily produced (during the 24 hours following) by a decreased or by an increased oxygen supply, and by greatly increased pressures of carbon dioxide. *c.* At this first day stage it was found very difficult to produce such abnormalities by means of *low* temperatures (1923b; 219 tests on embryos in eggs obtained *prematurely*; 388 tests on usual embryonic stages). *d.* It was made practically certain (1921a; and unpublished data) that unequal thickness of the egg shells markedly varies the permeability of the shells to oxygen in quite the same way that it restricts the loss of water vapor from within. Thus different embryos are subjected to unequal oxygen pressures though all *eggs* are exposed to atmospheres of normal oxygen content. *e.* When very high pressures of oxygen are used during the stage at which hemoglobin normally begins to form (nearly 2.0 days) the amount of the pigment formed is greatly diminished; and in many of the resulting dwarfed embryos its formation is almost or quite completely inhibited during the 2–4 days which they may survive the (24-hour) period of treatment (1921b). Older embryos subjected to very high oxygen pressures usually show the presence of hemoglobin in their amniotic fluid. In tests made on young chick embryos Stockard (1914) had noted a reduction of hemoglobin in some eggs treated with anesthetics; and Buchanan (1926) later obtained the same in embryos treated with HNC. *f.* In the study of a limited number of chick embryos we found (1924) that in general they respond to altered pressures of oxygen in quite the same way as do the embryos of the dove and the pigeon. *g.* A direct experimental test of the relation of changes in developmental rate to the formation of abnormalities and monsters—a test made for the first time in birds (Riddle, 1923b)—proved that various abnormalities arise not only from *retardations* (Dareste, Stockard, Child, Hyman), but also from accelerations of developmental rate. *h.* In one of these studies (1924) the necessary gaseous environment of the bird embryo of practically all ages was investigated; there the special importance to respiration of the first respiratory surfaces and of the early appearance of hemoglobin and a circulation, were subjects discussed at length.

These facts make it certain that during the first three days of development bird embryos are more injured by excess oxygen than at any later period of development or of life. This is precisely the period when the embryo is most nearly without mechanisms,[1] or is only beginning to de-

[1] The thinness of the blastoderm, i.e., its very restricted depth, is to be regarded as the very first structural adjustment to the gaseous environment.

velop mechanisms, of respiratory adjustment. During all later stages the effective oxygen supply to the tissues can be adjusted by rate of heart action, by diminution or increase of blood pigment, and doubtless by some advantageous responses in the rate of increase of special respiratory surfaces (area vasculosa, chorion, yolk sac, allantois); but until the last half of the second day (dove, pigeon) none of these mechanisms exist, and the third and fourth days find all these mechanisms developing at highest speed —quite out of proportion to other organ systems. Indeed, the most obvious and characteristic feature of avian embryology is the rapidity with which a complete respiratory mechanism—involving an elaborate efflorescence of temporary (respiratory) surfaces—is established during the first three or four days.

The earliest days of incubation are marked off from the total period in still other ways. During the first three days the chemical transformations within the entire egg of the chick are predominantly endothermic in nature, becoming exothermic only in the fourth day (Bohr and Hasselbalch, 1903). In these early days relatively most nitrogen is excreted in the form of ammonia (Needham, 1929). Tomita (1921) showed that the concentration of lactic acid in the hen's egg reaches its maximum on the fifth day and then rapidly decreases; Needham (1925) has suggested that only at this later stage is the enzyme which decomposes lactic acid present in sufficient amount. Tomita's curves for amount of lactic acid in total egg contents strikingly simulate Payne's curve for prenatal death in this same period. Again, it is during this early development only that the respiratory quotient indicates that carbohydrate is being burned by the embryo; Bohr and Hasselbalch found a rapid decline in the R.Q. from 0.9 on the fifth day to 0.71 on the ninth and thereafter. Certainly these related facts and conditions are also closely bound up with proper and effective tissue ventilation during this early stage of development.

Water loss as a cause of death at or near the end of incubation. First of all, it can be said that the avian egg is the sum of many engineering devices which attempt to provide an adequate food and water supply for a prospective embryo. In a consideration of the evolution of terrestrial vertebrates Gray (1928) has called attention to some of the egg's devices for the conservation of water. He noted that the egg shell is relatively impervious to vaporized water. Also, that the water-rich albumen is a colloid; if crystalloid, the embryo (within a yolk membrane much more permeable to water than to salt) would be able to take only a fraction of this water because of the increasing osmotic pressure external to the yolk membrane. Similarly, Fiske and Boyden (1926), and Needham (1929) have pointed out that sauropsidan embryos save large quantities of water by the simple device of excreting insoluble uric acid instead of soluble urea. "Only 20 per cent of the water in the egg need be set aside for handling uric acid." Needham

also calculated, from the data of Murray (1926), that the chick embryo gains about 2 grams of extra "metabolic water" by the burning of stored fat. Much less metabolic water would arise from the burning of protein or carbohydrate. It may be stated further that this yolk fat is burned chiefly near the end of incubation, and thus this supply of metabolic water is obtained at precisely the time that other water supply is most depleted. Finally, Wladimirow (1926) has shown that the embryo produces an as yet unidentified acid which diffuses into the egg white, slowly brings it to the isoelectric point, and thus releases water only gradually from the colloidal albumen.

In the case of a certain species of pigeon (genus and species ?), obtained from the Orinoco region of Venezuela and provisionally called Orinocans, we have found that only very rarely do the eggs contain enough moisture to permit hatching in dry nests in our colony houses. We have been able to hatch several eggs by making nests of sand or sod and pouring water daily into them. Having spent a summer in the native habitat of this bird I happen to know that the summer season is there characterized by daily rainfall. These eggs are doubtless adapted to hatching in the moist hot atmosphere of the Orinoco region. This species invariably lays only a single egg—not two eggs—per clutch. From three such females bred in our colony we have obtained 90 eggs. Altogether, we have 29 hatches; 40 dead embryos—*twenty-four* of which died during the final two days of incubation; 15 were infertile; and 6 were broken. The large air cavities of all of these eggs plainly showed a too rapid loss of water. It is entirely evident that most of the prenatal deaths observed by us in this species is due to the inability of the eggs to conserve water when incubated in our climate.

The relation of inadequate egg shells and rapid loss of water to embryonic death at or near the end of incubation in ordinary doves, pigeons and fowls is, however, a matter of difficulty and real confusion. Riddle (1921) could show that some individual doves which occasionally produce thin-shelled eggs produce also other quite thick ones and that a high proportion of prenatal death occurred in both kinds of eggs. In the case of some soft or thin-shelled eggs it was of course obvious that death must soon occur, since such eggs might show a loss of water 84 times greater than an egg with a normal shell. We concluded, however, that "an unknown and more deeply seated cause is responsible for both the occasional inadequate shells and the numerous early deaths." Dunn (1924) has examined this same situation in the egg of the fowl, and with rather similar results. He found, however, only a very small group of fowls whose eggs showed abnormally high rates of water loss and "were also marked by high mortality rates of their embryos." He observed in fowls, as we had earlier found in doves and pigeons, that there is a rather wide range of

evaporation rates which may be called normal; and within this range a particular rate of loss is not measurably associated with a particular rate of mortality. Nevertheless it is a well-known fact that some or many of the hatchable embryos in these "normal" eggs will not hatch if moisture is not supplied to the incubator. This clearly indicates that a fatal rate of water loss is close at hand for many eggs with apparently normal shells. Some current methods of supplying extra moisture (wet sand, etc.) to fowls' eggs, and the tests of Lippincott and de Puy (1924) in placing such eggs in shallow pans of water, seem to indicate the practicability of such means for increasing the number of hatches from eggs having high rates of water loss. Some eggs having quite low rates of water loss would probably have their enclosed embryos killed by this same procedure. Certainly, by supplying extra moisture to nests and eggs we have successfully hatched some dove eggs (in addition to the Orinocans) with high rates of water loss—eggs containing embryos which otherwise would surely have died during the last two or three days of incubation.

Discussion. No tests have thus far been made of the possibility that variable proportions of urea and uric acid are excreted—and therefore variable quantities of *water made unavailable within the egg*—depending upon the nature and adequacy of the shell. It is conceivable that the relative amount of these two nitrogenous excretions of these (cold-blooded) embryos is modified by variation in the oxygen pressure, or even by an unusually rapid loss of water from the egg contents. There are no adequate tests of the optimum humidity for each of the several stages of development; nor of how these optima are related to shell permeability. There are no complete answers to the query as to the number of abnormalities and malpositions of avian embryos which are actually produced by unfavorable oxygen pressures—or with these variously combined with different and varying temperatures; nor, indeed, of a possible connection between malposition and high rate of water loss. We have as yet only fragmentary knowledge of the chemical and physical condition—and changes of condition—of even the circumscribed contents of the egg. We seem to be still, as for the past hundred years, at or near the beginning of experimental investigation of the bird embryo.

SUMMARY

The age distribution of 2010 dead embryos, from three very distinct kinds of doves and pigeons, is figured and described. This age distribution in three newly studied groups, like that previously known in the common fowl only, shows two distinct peaks or periods of high death rate. Despite the variable length of the incubation periods of these four species the periods of maximum mortality occur at essentially equivalent pre-hatching stages in all cases; the death rate of the first period attains a maximum at

the third or fourth day, and the maximum of the second period—irrespective of the total length of the incubation period—includes the final two or three days of incubation. These two peaks of prenatal mortality are therefore probably characteristic of avian reproduction generally.

The excess mortality at these two periods seems to be only partially accounted for by the action of lethal factors and ordinary incubation conditions.

It is suggested that an important part of the mortality of the first period is due to failures in respiratory adjustment. This suggestion is shown to be supported by a wide survey of facts obtained from many sources, and hitherto unconsidered in their relation to this problem.

It is indicated that a notable share of the excess mortality that occurs immediately preceding hatching is ascribable to an inadequate water supply at this period. The many devices of the egg to obtain and to conserve its water supply are discussed.

The building of each individual egg of the bird is an engineering problem—and each problem is executed separately. Usually the finished structure permits the success of complete development; more frequent failures attend the arrangements which should provide the prospective embryo with a suitable gaseous and aqueous environment.

It is pointed out that the very device (thick shells) which is utilized by eggs to prevent water-loss—and avoid death in the final period—may too greatly affect respiratory exchanges and thus cause death in the early period. Many avian eggs are incompletely adapted vehicles of reproduction, and some good and fit germs are unable to survive.

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THE EFFECT OF LIVER ON THE BLOOD SUGAR LEVEL AND ON THE SUGAR EXCRETION OF DEPANCREATIZED DOGS

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Received for publication April 28, 1930

In two recent articles, Blotner and Murphy (1), (2) have reported that the administration of liver, or material made from liver, to diabetic patients produces a lowering of the fasting blood sugar level in certain of these patients. These investigators have reported that the substitution of raw pancreas for liver is not effective in lowering the blood sugar. This result leads them to suggest that the insulin present in the liver is not the agent responsible for the results they obtain. Murlin, Pierce, and Gregg (3), however, impressed by the action of certain constituents of liver in favouring the absorption of insulin from the alimentary tract, are inclined to the belief that the effect of liver feeding in diabetes reported by Blotner and Murphy is due to the insulin which the liver contains.

As facilities were available in this laboratory for observations on depancreatized dogs, it was decided to study the effect of liver feeding on the blood sugar and the sugar excretion of these animals. We have been unable to demonstrate any beneficial effect of liver on the condition of diabetic animals.

METHODS. Three series of experiments were carried out. In two series, healthy depancreatized dogs which had received minced pancreas and insulin in addition to lean beef muscle and sucrose for at least two weeks after pancreatectomy, were used. In the first series the diet of the animals was changed from the one outlined above to one consisting of weighed amounts of beef muscle, sucrose, and sodium oleate. Insulin was administered twice each day in amounts adjusted to permit a moderate glucosuria. After from three to six days under this regimen fresh beef liver was substituted for the lean meat and sodium oleate of the diet. The amounts of sugar and insulin administered were kept constant throughout the experiment. After periods of from three weeks to three months on the liver-containing diet the animals again received the control diet for several days. During the whole experiment, with the exceptions indicated in chart I, fasting blood sugar, sugar and nitrogen excretion values were determined daily.

In the second series the animals were placed on a diet of lean beef muscle and sucrose, and enough insulin was administered to keep them in good con-

dition. During the seventeen hours preceding each experiment the animals received no food or insulin. In some cases the sucrose was eliminated from the diet and no insulin was administered for an additional period of forty-eight hours before the experiment. The experiment which is the repetition of Blotner and Murphy's (2) procedure with diabetic patients, consisted in determining the effect of the administration of from 125 to 300 grams of fresh beef liver on the blood sugar of the animal. Blood samples were secured just before the liver was administered and at three hour intervals afterwards for a period of from nine to twelve hours. In the control experiments the procedure was the same but no liver was administered.

In the third series, experiments were carried out in which the effects of lean beef muscle and of beef liver on the blood sugar of normal dogs were studied.

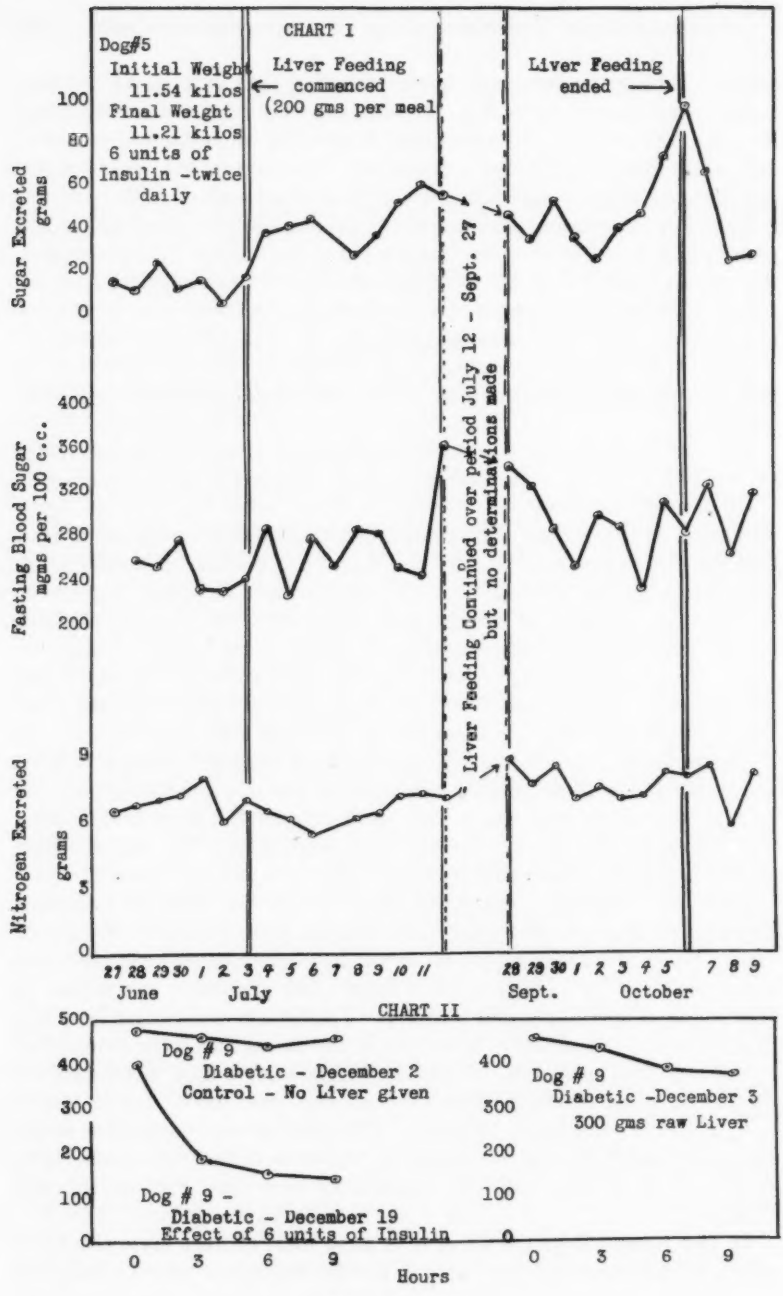
The blood and urinary sugars were determined by the Shaffer-Hartmann (4) method. Nitrogen was estimated by the macro-Kjeldahl technique.

EXPERIMENTAL RESULTS. *Series I.* Three short experiments were performed in this series. The control diet consisted of 150 grams of lean beef muscle, 50 grams of sucrose, and 5 grams of sodium oleate per meal. Two meals per day were given. The experimental diet was 50 grams of beef muscle, 50 grams of sucrose, and 100 grams of liver. Four units of insulin were administered twice daily throughout the period of observation. No evidence of any effect of the liver feeding on glucose utilization was secured. The amount of sodium oleate added to the control diet was based on a 5 per cent fat content of liver (Blotner and Murphy, 2).

On two other animals the effect of more prolonged feeding with liver was studied. In these animals liver feeding was commenced on July 3rd and determinations were made daily until October 6th when liver feeding was discontinued and the control diet substituted. The results were similar in the two cases and the data for dog 5 are shown in chart I.

Series II. In series I the object was to follow the effects of prolonged feeding with liver on the blood sugar and the sugar excretion of diabetic dogs. Series II was planned to study the immediate effect upon the blood sugar produced by a single meal of liver. The procedure was similar to that followed by Blotner and Murphy. The fasting blood sugar level was determined after a period of seventeen hours during which the animal had received no food or insulin. A relatively large meal of liver was then given and blood sugar determinations were subsequently made at intervals of 3, 6, 9, and in some cases, 12 hours. The controls were carried out under the same conditions as above except that no meal of liver was given. The results of the control and test experiments were similar in all essential details.

In two normal dogs the effect of a large meal of liver upon the blood sugar, after seventeen hours' fasting, was determined as above. Similarly



Charts I and II

the effect of a control meal of lean beef muscle was studied. The blood sugar values at 3, 6, and 9 hours after the meal were similar in the two cases.

Also two experiments with controls were performed on depancreatized animals which had been placed upon a diet of lean meat only and had received no insulin for a period of sixty-five hours before the beginning of the experiment. Typical results are plotted in chart II.

Under similar conditions as regards previous food and insulin the effect of the administration of 6 units of insulin was studied in the same animals. The results in one animal are plotted in chart II.

Discussion. Impressed by the favourable results reported by Blotner and Murphy (2) from the administration of raw liver to diabetic patients, we have performed extended experiments in order to study the effect of liver feeding upon the blood sugar level and sugar excretion of completely depancreatized dogs, and upon the blood sugar level of normal dogs.

We considered diabetic animals better test objects than diabetic patients because of the readiness with which the diet and available insulin can be accurately controlled. These animals are much more sensitive to any blood sugar reducing substance than are most human diabetics.

In the first series in each of the five experiments performed, the blood sugar level during the control period when no liver was given was, on the whole, lower than the blood sugar levels obtained during the course of the liver feeding.

In the second series of experiments, designed to study the immediate effects of the ingestion of fresh liver on the blood sugar of diabetic dogs, the results were also negative.

Since the patients studied by Blotner and Murphy still retained the pancreas it was thought possible that their favourable results might be due to the existence in liver of some substance which acted either directly or indirectly upon the "islets of Langerhans", stimulating them to greater activity and resulting in an increased endogenous supply of insulin. If this were the case our animals, retaining no pancreas, could not possibly yield positive results. Consequently it was decided to try the effect upon the blood sugar level of a meal of liver given to a normal dog and to compare this with the effect, if any, produced by a meal of beef muscle given under the same conditions. Only at the 3 hour period was the drop in the blood sugar level in the test experiments greater than in the controls, whereas at the 6 and 9 hour periods the blood sugar level actually rose above the fasting level when liver was given and fell in the case of the controls. Therefore, these results provide no evidence either for a blood sugar lowering principle present in the liver or for the presence of any principle which exerts a stimulating influence upon the "islets of Langerhans" of the pancreas of normal animals. The possibility exists that partially

depancreatized dogs, whose tissues presumably contain less than the optimum amount of insulin, might be better test objects. In our opinion this possibility is very faint.

The results in chart II show the effect of the administration of 6 units insulin to an animal after it had been subjected to the same treatment as above with regard to previous diet and administration of insulin. Blotner and Murphy have stated—"we feel that 180 grams of liver will have an effect upon the blood sugar of certain diabetic patients equal to that of from 10 to 15 units insulin." Whereas we failed to obtain any significant reduction in the blood sugar level of depancreatized dogs following the ingestion of what is relatively a much larger quantity of liver, we found a marked reduction following the subcutaneous injection of a smaller amount (6 units) of insulin. These results render the suggestion of Murlin, Pierce, and Gregg that Blotner and Murphy's findings might be due to the absorption of insulin contained in the ingested liver, highly unlikely.

The results of all these experiments throw doubt upon the suggestion of Blotner and Murphy (2) that liver contains "a blood sugar reducing substance active when taken by mouth, non-toxic, and with an effect on the blood sugar concentration similar to that obtained with insulin." If there were such a substance present in liver and acting directly as a blood sugar reducing substance, there is no reason why it should not give positive results when administered to totally depancreatized dogs. The animals were in excellent physical condition as a result of previous post-operative treatment with insulin, the liver was eaten with relish, and as a result of very high blood sugar levels they were extremely sensitive to blood sugar reducing substances.

SUMMARY

No evidence has been obtained that the ingestion of raw liver lowers the blood sugar or sugar excretion of depancreatized dogs or the blood sugar of normal dogs. The findings of Blotner and Murphy that liver feeding produces favourable effects on diabetes mellitus have not been substantiated by observations on depancreatized dogs.

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SALIVATION IS UNNECESSARY FOR THE ESTABLISHMENT OF THE SALIVARY CONDITIONED REFLEX INDUCED BY MORPHINE

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Received for publication April 30, 1930

In the conditioned reflex induced by repeated injections of morphine described by Collins and Tatum (1925) and studied quantitatively by Kleitman and Crisler (1927) it was shown that the impulse causing the unconditioned response does not need to come to the reflex center over an afferent path. In this case the stimulus, morphine, apparently acts directly on the reflex center. The afferent part of the unconditioned arc, then, is not necessary for the development of a conditioned reflex. This paper contains evidence tending to show that the unconditioned effector response is not necessary for the establishment of a conditioned reflex, and that, therefore, the total efferent limb of the unconditioned arc is not indispensable for the formation of the salivary conditioned reflex.

METHOD. The experiments are of two types. The first group depends upon the formation of the salivary conditioned reflex while the unconditioned effector response, the actual secretion of saliva, is prevented. The second group shows that the conditioned reflex is abolished, even when the effector response is caused to continue unconditionally.

Salivary fistulas were made by transplanting Wharton's duct, as described by Kleitman and Crisler, in five dogs and the saliva was collected in the manner described by them. Two or three experiments were run on each dog.

To check the contention of Mulinos (1929) that atropine can develop a salivary conditioned reflex we gave our animals 2 mgm. doses of atropine sulfate injected daily subcutaneously for 30 days. We found no evidence of the establishment of a conditioned secretion within that time. If such a conditioned reflex is possible its appearance seems to be too slow to complicate our results.

In the first group of experiments the animals received from 0.5 to 2 mgm. of atropine sulfate subcutaneously ten minutes before being placed in the stand for the test. The animals remained in the stand for ½ hour and then received 30 mgm. of morphine hydrochloride subcutaneously, after which they remained in the stand for a 15 minute post injection period. After

from 7 to 9 days, the atropine injections were discontinued, the animals still receiving morphine after ½ hour in the stand and remaining for the post injection period.

In the second group of experiments a conditioned reflex was established and continued until the secretion rate was practically constant from day to day. Pilocarpine hydrochloride (10 mgm. doses) was then substituted for the morphine and the disappearance of the reflex observed.

In some of these animals the second part of the experiment followed the establishment of the conditioned reflex in spite of the atropine. But in two of them the reflex was established in the usual manner, in which the formation followed the S-curve described by Kleitman and Crisler.

Results. It was found that there was no secretion of saliva, conditioned or unconditioned, following the injection of morphine if the animals were given atropine before being placed in the stand. After a time long enough to produce a fully formed conditioned reflex had elapsed (7 to 9 days as determined in previous experiments), and the atropine injections were discontinued, the animals secreted as much saliva as at any subsequent time. The conditioned reflex was here established in spite of the absence of any effector response, conditioned or unconditioned. See figure 1.

When a second reflex was established in the absence of atropinization, the first part of the S-curve, the part of increasing acceleration, was absent regardless of whether the first reflex had been developed in a normal or in an atropinized animal.

In the second group of experiments, in which after the animals had come to secrete a constant amount of saliva every day, and in which pilocarpine was then substituted for the morphine, the conditioned secretion of saliva disappeared within the normal time. See figure 2.

Discussion. During the early part of the experiments the atropine dosage used was 2 mgm. One of the dogs developed definite signs of cortical stimulation presumably from the added effects of the atropine and morphine, because he failed to show these effects on either atropine or morphine given separately. He showed periodic convulsions, failed to respond to his name, attempted to bite whenever touched, and at times was almost unmanageable. He ran into objects, apparently having lost his vision. He ate very little, and his water intake was noticeably decreased. On the basis of these symptoms the dosage was successively cut to ½ mgm. which proved sufficient to prevent the secretion of saliva, either conditioned or unconditioned.

In 1914 Doeve reported a paradoxical secretion of saliva after repeated injections of atropine. In our experiments this does not result within a month. Our dogs were tested before any conditioned reflex had been established. We found, however, that if we tried it on dogs which had had

previous conditioned reflex experience, and placed the animals in the stand before injecting the atropine, we sometimes obtained several tenths of a cubic centimeter of saliva before the atropine produced its inhibiting effect. We interpreted this as a residual secretion from the previous conditioned reflex experience. We therefore adopted the plan of giving the injection of atropine 10 to 15 minutes before placing the animals in the stand when we were testing for the establishment of the conditioned reflex in the absence of the effector response. It is to be remembered, however, that when we were testing for the establishment of a conditioned reflex from the injection of atropine, the animals were in the stand daily for ½ hour before

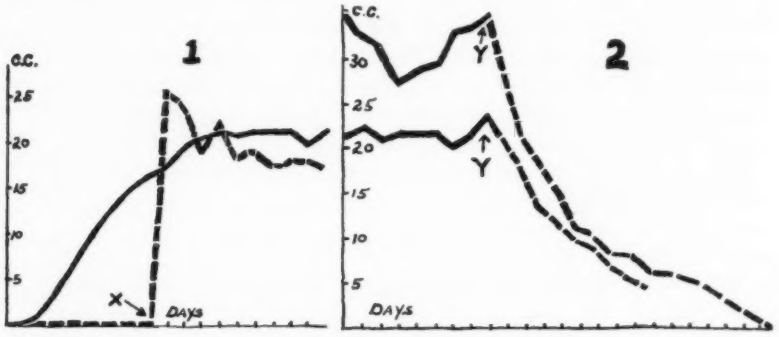

Fig. 1. A comparison of the reflex established normally and during atropinization in the same dog. The solid curve shows the reflex induced normally, exhibiting the characteristic S-curve. The dotted curve represents the establishment of the reflex during atropinization. Atropine was discontinued at *X*.

Fig. 2. Curves of two dogs showing a typical extinction of the reflex when pilocarpine is substituted for morphine. The solid portions of the curves represent the plateau secretions of conditioned dogs. The dotted portions represent the extinctions after substituting pilocarpine for morphine at *Y*.

the drug was given and at no time was there a conditioned secretion of saliva as measured by our method.

Two obvious possibilities are suggested for this paradoxical secretion of saliva which Doeve reports. In those cases where it occurs there may be anomalies in the innervation of the salivary glands. This may not be so improbable in light of the paradoxical case of the sweat glands with reference to innervation and the action of atropine. Or it may be that with optimum doses of atropine for demonstrating it, the drying of the mouth in some way acts as a stimulus which after a time leads to the paradoxical secretion as a sort of defense mechanism against drying.

From a study of the conditioned secretion curves it is apparent that the reflex is established in the absence of the effector response. It is supposed

that the changes in the nervous system are very similar whether the effector response is allowed to appear during conditioning or not. As primary evidence for this fact is the similarity of the "plateau curves" after conditioning in each case. Also there is the absence of the first part of the S-curve during a second establishment of the conditioned reflex. That is, the changes in the nervous system when the effector response is absent, must be at least near enough normal that the entire process does not need to be repeated during a second establishment of the conditioned reflex.

The second part of the experiment shows that the continuation of the effector response by a drug, pilocarpine, which of itself can not cause a conditioned reflex (Kleitman, 1927; Crisler, 1928) is not enough to prevent the extinction of the conditioned reflex when the stimulus causing conditioned secretion, morphine, is discontinued. Whether or not this extinction process is retarded by pilocarpine can not be determined from the data presented, because, as Kleitman and Crisler have shown, the curves of extinction are by no means as constant as the curves for the establishment of the conditioned reflex because of the great individual variation in different animals.

CONCLUSIONS

1. Unlike morphine, atropine does not cause a conditioned secretion of saliva.

2. Morphine can induce a typical salivary conditioned reflex when the actual secretion of saliva is prevented by atropine. Hence the effector response is not indispensable for the establishment of a typical salivary conditioned reflex showing that less than the entire efferent limb is necessary for the conditioning process.

3. Continuation of the effector response by pilocarpine after a salivary conditioned reflex has been induced by morphine does not prevent the extinction of the conditioned reflex. Hence elicitation of the effector response independent of the nervous system does not appreciably change the course of the extinction process.

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THE MECHANISM OF EPINEPHRINE ACTION

VII. Changes in the Glycogen, Lactic Acid and Phosphate Content of Muscle

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Received for publication May 5, 1930

A decrease of glycogen was noted when whole rats (with the exception of liver and viscera) were analyzed 3 hours after an epinephrine injection (1). Sahyun and Luck (2) found without exception a marked reduction of muscle glycogen in unanesthetized rabbits 1 to 42 hours after subcutaneous injection of about 0.5 mgm. of epinephrine per kilo. The animals were killed by stunning, the sciatic nerve was cut and the sartorius muscle excised. Two and one-half to 3 minutes elapsed between stunning the rabbit and dropping the muscle (cut longitudinally) into hot 60 per cent KOH. Eadie (3) performed 2 experiments on amytalized cats. One and one-half hours after subcutaneous injection of 1 mgm. of epinephrine per kilo he found in one animal a marked reduction, in a second one hardly any change in muscle glycogen. No conclusions should be drawn from two observations with inconsistent results. For a full discussion of the older literature reference is made to two recent publications (1), (4b).

Evidence of an indirect nature also points to a decrease of muscle glycogen after epinephrine administration. After a subcutaneous injection the blood lactic acid rises in unanesthetized cats, rats and rabbits (4). In the last named species a lactic acid content higher in venous than in arterial blood of the leg was found during epinephrine action. In order to dispose of any objection as to dosage (the rate of absorption from the subcutaneous tissue being an unknown quantity) intravenous infusions at constant and very slow rates were made in unanesthetized rabbits (5) and men (6). The blood lactic acid (and the blood sugar) rose shortly after the start of the infusion and remained elevated as long as the infusion continued.

The experiments on record in which direct glycogen determinations in muscle were made after epinephrine injection are open to several objections: 1. Davenport and Davenport (7) by determining lactic acid in muscle, showed that glycogenolysis is unavoidable unless the muscle is excised from the living animal. The nervous discharge following stunning, decapitation, etc., always leads to a breakdown of muscle glycogen. In

the work of Cori and Cori and Sahyun and Luck, the standardization of procedure adopted probably makes this loss quite uniform. 2. The question may be raised as to whether or not the decrease of muscle glycogen after epinephrine injection is due to excessive movements or restlessness of the animals. The experiments in which a rise in blood lactic acid occurred in amytalized animals seem to answer this question in the negative. 3. Whenever a decrease in muscle glycogen was demonstrated by direct analysis, epinephrine was administered subcutaneously, thus leaving the question of dosage unsolved.

As will be seen in the following work these three sources of error were avoided. Since it is known that phosphocreatine plays an important rôle in muscle metabolism, determinations of this substance and of inorganic phosphates were also undertaken.

EXPERIMENTAL. *I. Muscle glycogen.* Rats weighing 130 to 160 grams after being kept without food for 24 hours, were given 7 mgm. of amytal per 100 grams of body weight intraperitoneally. Fifteen to 20 minutes after the injection one gastrocnemius muscle was extirpated by severing it at the tendons. This procedure takes only a few seconds. By clamping the wound, bleeding could, as a rule, be completely avoided. The muscle was immediately introduced into a tared centrifuge tube containing 1 cc. of 60 per cent KOH. After weighing, the tube was kept for 3 hours in a boiling water bath. The glycogen was precipitated, washed and hydrolyzed in the same tube. Osterberg (8), using a similar procedure, showed that as little as 0.02 mgm. of glycogen can be recovered quantitatively. In the present experiments 4 to 6 mgm. of glycogen were available for each determination. After neutralizing the glycogen hydrolysate with KOH, the sugar was determined by means of Hagedorn and Jensen's (9) method. In several experiments the non-sugar reducing substances of the hydrolysate were determined after removal of the sugar with copper sulfate and lime. Since they constituted only 4 to 5 per cent of the total reducing substances no attempt at separation was made in the experiments recorded in table 1.

Immediately after extirpation of the first muscle epinephrine was injected subcutaneously or an intravenous infusion was given at a constant rate, lasting for 30 minutes (table 1). The controls were given salt solution in a similar manner. The 2nd gastrocnemius muscle was extirpated 30 minutes after the subcutaneous injection or at the end of the infusion and treated in exactly the same manner as the first. After the sampling of the 2nd muscle the rats were killed and blood was collected. Lactic acid and sugar were determined in blood filtrates prepared according to Folin-Wu and to Somogyi's (10) directions respectively, the former by Friedemann and Kendall's (11) the latter by Hagedorn and Jensen's method. Table 1 shows that in 6 control experiments the average glycogen content of the

2 muscles agreed very well. With one exception (rat 5) the glycogen content of the right and left gastrocnemius muscle of the same rat showed differences of less than 3.5 per cent. Since the second muscle was removed 30 minutes after the first one, this confirms Hinsey and Davenport's (12) contention that amytal has no effect on the glycogen content of resting muscles. Blood sugar and lactic acid values showed little variation and are within the range usually observed in fasting unanesthetized rats. After epinephrine injection, irrespective of the mode of application of the hormone, the muscle glycogen content was, without exception, significantly reduced. At the same time blood sugar and lactic acid were definitely elevated.

TABLE 1

Effect of epinephrine injections on muscle glycogen

The rats were under amytal anesthesia. Muscle II (and blood) was taken 30 minutes after muscle I.

CONTROLS				EPINEPHRINE			
Glycogen in gastrocnemius		Blood lactic acid	Blood sugar	Glycogen in gastrocnemius		Blood lactic acid	Blood sugar
I	II			I	II		
mgm. per cent	*mgm. per cent*	*mgm. per 100 cc.*	*mgm. per 100 cc.*	*mgm. per cent*	*mgm. per cent*	*mgm. per 100 cc.*	*mgm. per 100 cc.*
596*	576	13.6	90	596‡	366	43.0	123
599*	597	20.4	118	546‡	455	32.5	129
687*	680	11.1	91	564‡	468	24.0	102**
564†	577	10.0	80	568§	474	25.9	142
490†	575	12.0	96	631§	524	34.2	128
578†	579	14.8	94	637§	522	22.3	127
Average..586	597	14.0	95	590	468	30.3	125

* 0.2 cc. of salt solution injected subcutaneously.

† 1.4 cc. of salt solution injected intravenously in 30 minutes.

‡ 0.2 mgm. of epinephrine per kilo injected subcutaneously.

§ 0.0005 mgm. of epinephrine per kilo per minute for 30 minutes.

** Muscle II and blood were taken 15 minutes after muscle I.

Epinephrine lowers the muscle glycogen at a rate of application which is far below the maximal rate at which this substance can, according to Cannon and Rapport (13), be liberated from the adrenals. The effect is an early one, observable 15 to 30 minutes after administration of the hormone and possibly sooner; it occurs in completely relaxed, anesthetized animals. The response to a subcutaneous injection or intravenous infusion was identical, though the amount given with the latter mode of application was only 1/13 of the amount injected subcutaneously. Recently it has been possible to determine fairly accurately the upper and lower limit of the rate of absorption of epinephrine from the subcutaneous tissue of rabbits

and rats (14). This rate was found to be well within maximal physiological limits. The subcutaneous form of application in the doses indicated may therefore be used with impunity in investigations designed to study physiological effects of epinephrine.

Assuming that the musculature amounts to 50 per cent of the body weight, the average loss of glycogen shown in table 1 would correspond to 61 mgm. per 100 grams rat. In previous work in which the glycogen content of the whole carcase was determined, an average of 57 mgm. of glycogen had disappeared 3 hours after a subcutaneous injection of epinephrine in fasting rats (1). Though no attempt has been made to explain the "utility" to the animal of the disappearance of muscle glycogen and rise of blood lactic acid after epinephrine administration, this can hardly justify a non-acceptance of the experimentally established facts (Cannon, 15).

A few words may be added concerning the amounts of glycogen present in the muscles of rats fasting for 24 hours. Macleod and collaborators (16) give 0.3 per cent as the average of 24 determinations. Since the average of 12 determinations in the present investigation is 0.588 per cent it was decided to follow Macleod's directions for sampling the muscles. When this was done the following values for glycogen of the gastrocnemius muscle were obtained: 0.355, 0.352, 0.423, 0.414, 0.325, average 0.374 per cent. It is evident that by decapitating and bleeding the animal and by cutting into the muscle large amounts of glycogen are lost, though by standardizing the procedure very consistent values can be obtained.

The evidence of Chaikoff and Weber (17) that epinephrine causes a conversion of fat into sugar in the pancreatectomized dog rests on two contentions: 1, that epinephrine does not cause a breakdown of muscle glycogen, and 2, that all the muscle glycogen present, even if it were mobilized, could not account for the extra sugar eliminated. Exception to the first contention has already been taken. Since the carbohydrate reserves of the body were calculated from glycogen determinations in muscles obtained after killing the animals—a procedure which has just been shown to lead a considerable loss of glycogen—exception must also be taken to the second statement.

2. Determination of muscle phosphates and lactic acid. Fiske and Subbarrow (18) found that in determining phosphocreatine, freezing the muscle in situ (Sacks and Davenport, 19) is not essential in the prevention of a breakdown of phosphocreatine, provided the first steps of the method are carried out in a cold room. This could be confirmed in the present work. The same is true for the prevention of glycogenolysis.[1] The following method was adopted. One group of amytalized rats was given a

[1] Doctor Davenport, in a personal communication, informed the writer that he has since come to the same conclusion.

subcutaneous epinephrine injection. The controls received the same amount of salt solution. The first gastrocnemius muscle was extirpated 18 to 21 minutes, the second one 36 to 45 minutes after injection. The muscle was weighed on a cooled watch glass in a cold room (5 to 8°C.) and introduced into ice-cold trichloracetic acid. Inorganic P and phosphocreatine were determined by Fiske and Subbarow's method (separation by precipitation with an alkaline solution of $CaCl_2$), lactic acid by Friedemann

TABLE 2

Effect of epinephrine on muscle phosphates and lactic acid

The rats were under amytal anesthesia. Muscle II (and blood) was taken 20 minutes after muscle I.

INORGANIC P IN GASTROCNEMIUS		PHOSPHOCREATINE P IN GASTROCNEMIUS		LACTIC ACID IN GASTROCNEMIUS		BLOOD LACTIC ACID	BLOOD SUGAR	LACTIC ACID IN LIVER
I	II	I	II	I	II			
Controls								
mgm. per cent	*mgm. per cent*	*mgm. per cent*	*mgm. per cent*	*mgm. per cent*	*mgm. per cent*	*mgm. per 100 cc.*	*mgm. per 100 cc.*	*mgm. per cent*
30.0	29.0	70.1	55.8					
30.8	28.4	55.0	48.5	13.0	11.0			
30.3	30.6	51.3	51.3	12.1	16.3	10.6	88	
31.0	18.2	57.4	60.3	9.5	8.4	7.7	77	14.2
30.8	26.5	57.7	65.2	13.2	10.7	12.4	91	30.2
20.2	22.0	63.3	65.1	12.4	13.5	9.2	78	18.2
28.8	25.8	59.1	57.7	12.0	12.0	10.0	90	20.8
0.2 mgm. of epinephrine per kilo subcutaneously 20 minutes before extirpation of muscle I								
18.9	23.1	54.9	47.6					
29.4	24.8	71.0	84.7	45.0	51.0			
29.9	26.6	44.5	53.5	32.4	39.2	23.0	141	
27.3	22.9	57.5	59.2	12.1	35.0	33.8	132	
14.1	21.9	63.6	56.1	34.0	49.4	34.9	141	42.2
18.8	19.3	61.0	57.6	44.7	67.4	43.2	175	41.4
23.0	23.1	58.7	59.8	33.6	48.4	33.7	147	41.8

and Kendall's method. Immediately after extirpation of the 2nd muscle the animals were killed and blood was collected.

Table 2 summarizes the experiments. The control group shows that occasionally considerable differences are encountered in the phosphate fractions of symmetrical muscles of the same animal. The values for both fractions fall within the range observed for other species by Fiske and Subbarow. Twenty and 40 minutes after epinephrine injection the

amount of inorganic phosphates in muscle is slightly depressed. A fall of inorganic P of blood after epinephrine administration has been observed by several investigators (Perlzweig, Latham and Keefer, 20). Since there is no difference in the phosphocreatine values in controls and animals injected with epinephrine, the sum of the two fractions is slightly lower in the second group. That the decrease in muscle glycogen after epinephrine injections has nothing to do with muscle contraction is also made probable by the fact that the phosphocreatine content of muscle is not affected by epinephrine administration.

Muscle and blood lactic acid values of the controls are of similar magnitude. Davenport and Davenport (7) obtained equally low values with their method of freezing in vivo and their conclusion that in the resting state the lactic acid concentration of blood is equal or slightly lower than that of the muscles, is supported. After epinephrine injection muscle and blood lactic acid were definitely elevated. Forty minutes after injection the muscle lactic acid was higher than 20 minutes after injection. Since the lactic acid content of blood and muscles tends to be in equilibrium, a high lactic acid value in muscle need not necessarily mean that the lactic acid originates there. That this is the case is made very probable by the simultaneous decrease of muscle glycogen.

SUMMARY

1. The glycogen content of the right and left gastrocnemius muscle, when extirpated at 30 minute intervals from rats under amytal anesthesia, was found to be of the same magnitude. When after extirpation of the first muscle epinephrine was injected either subcutaneously or by means of an intravenous infusion at a constant rate, a definite reduction in the glycogen content of the second muscle was always encountered.

2. Twenty and 40 minutes after epinephrine injection the phosphocreatine content of muscle was practically unchanged, the inorganic phosphates showed a slight decrease and the lactic acid content of muscle and of blood was markedly elevated.

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Addendum. While this article was in press, three papers came to our notice dealing with the influence of epinephrine on muscle glycogen. Eadie (1930. This Journal, xciv, 69) finds that small doses of epinephrine, i.e., 0.1 mgm. per kilo subcutaneously, always lead to a decrease in muscle glycogen of rats, while large doses (1 mgm. per kilo) are supposed to have an uncertain effect. The animals were killed by decapitation previous to the removal of muscles for glycogen analysis. In the cat there is according to Eadie no change in muscle glycogen following the injection of small or large doses, a fact which is ascribed by this author to a definite species difference. Some of Eadie's results are not in accord with the following. Zimmermann (1929. Biochem. Zeitschr., ccvi, 369) who excised the muscles from the living animal under anesthesia, found in numerous experiments that 1 mgm. (i.e., the large dose used by Eadie) as well as 10 mgm. of epinephrine per kilo invariably caused a decrease in muscle glycogen of rats. In a paper entitled, "The effect of adrenalin on muscle glycogen," which the authors were kind enough to send us before publication, Corkill and Marks recorded a decrease in muscle glycogen in the spinal preparation of the cat in each of the experiments performed.

A NOTE ON DETERMINATION OF CIRCULATION TIME

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Received for publication May 6, 1930

The literature on circulation time is rather voluminous, and it is not feasible to review here the various determinations of the time required by the blood to complete the circuit of the vascular system by injecting a solution into a vein and detecting the same in the corresponding vein of the opposite side. Suffice it to say that Vierordt (1858), for example, using the potassium ferrocyanide method obtained an average circulation time of 16.32 seconds for the dog.

Stewart, Rogoff and Gibson (1916) in their studies on liberation of epinephrin from the adrenal glands determined the circulation time from the level of the adrenals to the carotid and to the eyeball by the methylene blue method. They found that the time required for the blood to pass to the eye was several seconds less than the latent period of the mydriasis produced by splanchnic stimulation. They then injected into the femoral vein 1.5 to 2.0 cc. of a 1:50,000 solution of epinephrin together with the methylene blue and noticed that mydriasis did not occur until a few seconds after the solution had reached the eye.

In the course of our studies on the duration of pupillary action of epinephrin and atropin (Koppanyi and Lieberson, 1930) we administered these drugs by various routes. When the drugs were injected into the left common carotid artery, the pupillary dilatation in the right eye occurred several seconds after the dilatation of the left pupil. The delay in the pupillary response of the right eye was explained as being due to the fact that the drug reached the left eye directly, while it had to make a complete circuit before reaching the right eye. The time interval, then, between the dilatations of the left and the right pupils, expressed in seconds, represents total circulation time.

The purpose of this study was to ascertain whether the pupillary response to drugs can be used to fix circulation time with a fair degree of accuracy.

METHODS. Cats, dogs and albino rabbits were used for these experiments. The drugs (epinephrin, procain, atropin and pilocarpin) were injected under local phenol anesthesia into the left common carotid artery

with a tuberculin syringe through a fine needle. In smaller animals (from one to three kilograms of body weight) usually no more than 0.5 cc. of solution was used so as to obviate any possible disturbance to the cerebral vascular bed. The observer in front of the animal recorded the pupillary response in both eyes following injection; while at times another observer took the heart rate during the period of the experiment.

Determination of total circulation time. The injection of 0.25 mgm. of epinephrin hydrochloride 1:1000[1] into the left common carotid artery of the cat was followed usually within one second by maximal dilatation of the left pupil, and from six to nine seconds later by dilatation of the right pupil. This time interval between the pupillary responses in the two eyes was taken to represent total circulation time. The blood carrying the epinephrin must have crossed both the pulmonary and a systemic circuit. The capillary bed of the systemic circuit in this case was that of the brain, since the results obtained by the injection of epinephrin into the common carotid artery were not modified by ligation of the external carotid artery. The following protocol illustrates this point.

April 26, 1930. Female cat, 2.72 kgm.

2:27 p.m. Injection of 0.1 mgm. epinephrin hydrochloride into the left common carotid artery.
Maximal dilatation of the left pupil—1 second following injection.
80 per cent dilatation of the right pupil—7.5 seconds following injection.
Circulation time—6.5 seconds.

2:30 p.m. Animal anesthetized with ether, and the left external carotid artery ligated.

2:48 p.m. Injection of 0.1 mgm. epinephrin hydrochloride 1:1000 into the left common carotid artery.
Dilatation of the left pupil—1.5 seconds following injection.
Dilatation of the right pupil—8.5 seconds following injection.
Circulation time—7 seconds.

The total circulation time was also determined by injections of the mydriatics: procain hydrochloride and atropin sulphate; and by the miotic: pilocarpin hydrochloride, into the left common carotid artery. The results obtained with these drugs parallel those obtained with epinephrin (see table 1). It should be noted, however, that pilocarpin reaching the left eye in high concentration produced miosis by parasympathetic stimulation, whereas having been highly diluted by a large volume of blood it produced fleeting *mydriasis* on the right side due to sympathetic sensitization (see Koppanyi, 1930).

The total circulation time was also determined in dogs and found to be about 14 seconds.

[1] All doses are expressed in terms of milligrams per kilogram of body weight. The words "per kilogram of body weight" are omitted to avoid repetition.

Control experiments on total circulation time. In order to eliminate the possible objection that the results obtained may partly be due to the passage of epinephrin to the right eye directly by some anastomoses in the carotid system, solutions of methylene blue were injected into the left

TABLE 1

Showing total circulation time as the interval between the pupillary responses in the two eyes following the injection of drugs into the left common carotid artery

DRUG	DOSE	CIRCULATION TIME	NUMBER OF HEART BEATS IN CIRCULATION TIME PERIOD
Cats			
	mgm.	*seconds*	
Epinephrin hydrochloride	0.25 (1:1000)	7	17
	0.25 (1:1000)	7	26
	0.25 (1:1000)	6	
	0.25 (1:1000)	9	
	0.10 (1:1000)	7	
	0.10 (1:1000)	7.5	
	0.05 (1:5000)	8	
Pilocarpin hydrochloride	0.25 (1:1000)	9	32
	0.25 (1:1000)	7	26
	0.25 (1:1000)	8	25
Atropin sulphate	2.50 (1:100)	8	24
	2.50 (1:100)	6	30
	2.50 (1:100)	8	
Procain hydrochloride	20.0 (1:20)	7	
Methylene blue (detected by blue discoloration of carotid blood)	25.0 (1:10)	6	
	15.0 (1:10)	7.5	
Dogs			
Epinephrin hydrochloride	0.25 (1:1000)	14	30
Pilocarpin hydrochloride	0.25 (1:1000)	13	29
	0.04 (1:1000)	15	

common carotid artery and recovered from the right carotid artery below the level of injection of the dye on the left side in from 6 to 7.5 seconds following injection.

To determine whether the exact systemic path which the injected drug

takes, besides the pulmonary circuit, affects circulation time to any appreciable extent, the capillary bed of the hind limb was substituted for the cerebral circuit by injecting the epinephrin into the femoral artery. In these experiments, mydriasis was noted in both eyes from 8 to 9 seconds after the injection. These results are comparable then to the determination of total circulation time by injection into the common carotid artery.

Determination of pulmonary circulation time. The injection of 0.1 mgm. epinephrin hydrochloride 1:1000 into the femoral vein was followed by mydriasis in from 3.5 to 4.5 seconds. Subtracting about one second from these figures to represent the possible latent period in the pupillary response, gives us the approximate pulmonary circulation time. Thus pulmonary circulation time is a little less than one-half of total circulation time.

Addendum. In three albino rabbits attempts were made to determine total circulation time using the constriction of the blood vessels of the ear as the biological index. The ears were painted with cedar oil and placed in front of an electric bulb to facilitate observation of the finer changes in the size of the blood vessels. The injection of epinephrin hydrochloride into the left common carotid artery did not produce as pronounced and immediate a change in the blood vessels as in the pupils. It usually took about four seconds until appreciable vasoconstriction was produced. This makes the method poorly adapted to measurement of circulation time. In one case vasoconstriction was detected in the left ear 3 seconds, and in the right 12 seconds following injection. If we allow 3 seconds as latent period of noticeable constriction of the ear vessels, the total circulation time of the rabbit would be about 6 seconds.

CONCLUSIONS

The foregoing results indicate that drugs eliciting pupillary responses may be used for determination of the lesser and greater circulation time. The results of Stewart, Rogoff and Gibson (1916) suggesting that the latent period of pupillary dilatation after epinephrin injection is several seconds were probably due to insufficient dosage, the deep anesthesia, or the impairment of the circulation accompanying surgical shock. The sharpness of the reaction and the simplicity of technique makes this "pharmacological" method of determining circulation time especially useful in classroom demonstration or student experimentation.

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AN EXPERIMENTALLY PRODUCED PREMATURE SYSTOLIC ARHYTHMIA (PULSUS BIGEMINUS) IN RABBITS

I. Its Nature and Agents which Produce It

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Received for publication May 17, 1930

This arhythmia appears in a number of Kratschmer's blood pressure tracings following the projection of ammonia into the nostril of rabbits and is described by him as an irregularity closely associated with pulsus bigeminus. It did not come on with the stimulation and frequently not until the blood pressure had returned to normal. Kratschmer states that it occurs in spite of vagotomy and after curarization.

In man Koblanck obtained an arhythmia from stimulation of a certain spot on the nasal septum opposite the middle concha. It is also well known that many tobacco smokers have premature systoles.

Koblanck's human observations led to some experiments by Koblanck and Roeder on dogs and rabbits in which a bigeminal pulse, or sometimes only a slowing of the pulse, was demonstrated when a certain spot on the nasal septum opposite the middle concha was stimulated electrically or mechanically. Vagotomy was said to remove the effect at first, but it reappeared with successive stimulation somewhat reduced. Sectioning one maxillary nerve through the maxillary bone removed the effect from the corresponding side but not from the opposite side.

MacWilliam described a slowed pulse resulting from the inhalation of chloroform in cats and some of his blood pressure tracings suggest a bigeminal pulse.

Levy and Lewis observed premature ventricular contractions in cats which inhaled 1 per cent chloroform vapor for 5 minutes.

Lohmann obtained extra systoles in dogs from prolonged stimulations of the vagus nerve.

It has also been shown that almost any direct irritation of a ventricle results in a premature contraction.

The work of Heymans and others on cardiac arhythmia will be considered in connection with the second part of this problem.

In a previous paper this arhythmia (fig. 2 C) was described briefly under the head of a pseudo-vagal reaction and during the first part of the previous

study the giant pulse waves which sometimes followed the inhalation of certain irritants were misinterpretated for a peripheral vagal reaction.

Nature of the arhythmia. In figure 1 A the apical beat tracing above and the carotid blood pressure tracing below are characteristic of many records taken from normal and operated rabbits at the time of insufflation of irritating vapors.

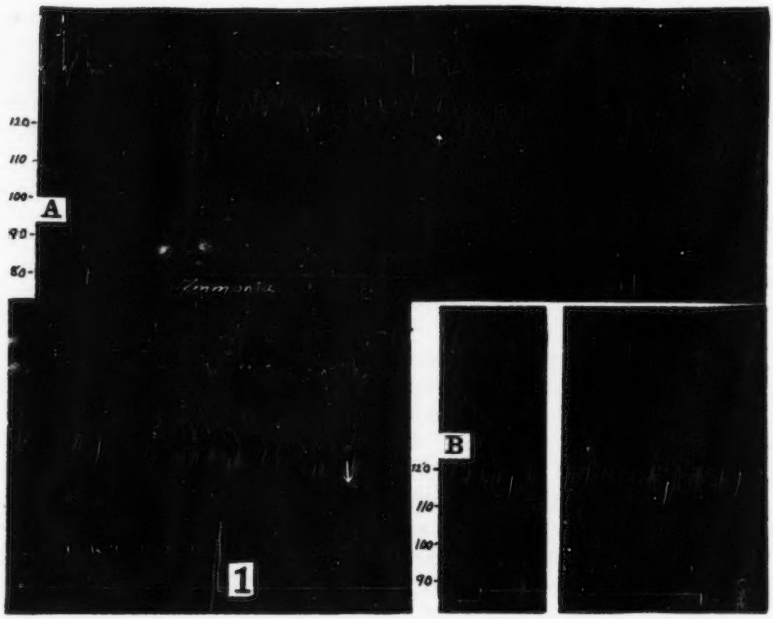

Fig. 1, A. Apical beat above and carotid blood pressure below during insufflation of ammonia into nostril of a tracheotomized "olfactory" rabbit. Time in seconds, ↑ indicates appearance of premature systoles and ↓ end of premature systoles.

B. Same animal and order of tracings as 1A—shows first and last portions of asphyxia graphs: omitted part represents 26 seconds. Observe absence of premature contractions.

In this rabbit all trigeminal supply to the nostrils was cut off by sectioning the maxillary and naso-ciliary nerves on both sides two days before the tests. The severance of these nerves was verified at autopsy. The animal was given a little less than a full anesthesia dose of veronal-sodium intravenously in divided doses and during the operation was kept under light ether anesthesia. The irritating vapor was forced into a nostril from a vaporizing bottle. Respiration took place through a tracheal cannula. A second cannula inserted through the crico-thyroid membrane and tied

above the thyroid cartilage not only prevented the vapor from entering the larynx but furnished an outlet, thus providing a current of concentrated vapor for stimulating the deeper portions of the nasal cavity during insufflation. The apex beat was taken by a tambour strapped to the thorax over the heart and connected by tubing to a Marey's tambour. This apparatus also recorded respiration. Blood pressure was taken from the right carotid by a mercury manometer.

In figure 1 A the arhythmia (bigeminal pulse) as indicated by an arrow appears after 14 seconds of insufflation. The apical beat tracing above discloses an arrest of respiration during the 21 seconds' interval of insufflation and for 7 seconds afterward. The duration of the arhythmia, indicated by two arrows, lasted for 27 seconds' some 20 seconds after insufflation was stopped and 12 seconds after respiration was resumed. Before the arhythmia appears the apex beats in the upper tracing exhibit a gradual slowing and strengthening and some body movement is shown directly before the arhythmia. Meanwhile the carotid tracing below reveals the immediate and pronounced rise in blood pressure and the gradual slowing and strengthening of the pulse described in a previous paper under insufflations of irritants.

In the bigeminal pulse portion of figure 1 A the first premature contractions in the apical beat tracing follow their respective normal beats much more closely than they do in the latter part of this tracing or than they do in other records. Consequently these premature contractions are followed by longer diastolic periods. The carotid tracing of figure 1 A likewise differs from the majority of bigeminal pulse tracings in that it demonstrates two types of bigeminal excursions. The giant excursions shown in the first part of the arhythmia are illustrative of *type 1* and correspond to the first bigeminal pulse beats in the apex beat tracing. These bigemina carotid excursions, including the very first one, are not only much higher than the preceding normal pulse waves, but they are approximately double their width. There is a very minute notch (often barely visible) close to their apices, which separates the normal pulse from the premature systole. The carotid blood pressure excursions of *type 2* shown in the latter part of the arhythmia in figure 1 A are much broader at their apices and the notch between the systole and the premature systole is much deeper. The difference between these two types is apparently dependent on the particular time in diastole of the precontraction. Its earlier appearance in type 1 results in a higher excursion.[1]

[1] In carotid pressure tracings when the rate of respiration is fast and conforms exactly to two pulse waves, as it sometimes does, it is difficult to discriminate between the respiratory effects and a series of bigeminal beats of the second type; hence it is advisable to take simultaneous thoracic tracings, which has been done in nearly all tests. If respiration is slightly slower or faster than two pulse beats, the pulse waves will change their position in a series of respiratory waves.

To illustrate the difference between the bigeminal pulse excursions just described and the superficially similar gigantic pulse of asphyxia, graph 1 B is introduced as representative of many tracings from several animals during constriction of the trachea. It is obvious that the excursions in both tracings of graph B, including the omitted portion of 26 seconds, exhibit no premature contractions. The carotid pressure (lower tracing) reveals only a very slow rise in blood pressure and a gradual slowing and strengthening of the pulse accompanying the increase in asphyxia together with a gradual return to normal after ventilation is resumed.

There should be little or no danger of confusing the bigeminal carotid pulse with the irregular carotid pulse obtained from peripheral stimulation of the vagus.

By palpation or opening the thorax the bigeminal pulse can readily be detected while being recorded.

Origin of the bigeminal pulse in the heart. To determine the atrial cycle at the time of this arhythmia, simultaneous apical beat, carotid pressure and venous pressure tracings from the superior vena cava or right atrium were taken of a number of bigeminal pulse arhythmias in five different rabbits.

The procedure of taking the vena cava tracings consisted of inserting a very long cannula filled with anti-coagulant into the right jugular vein until it extended past the subclavian vein. This cannula was connected to a specially made water manometer by tubing filled with the anti-coagulant. A film of oil added to the surface of the water prevented the cork bearing the rider from sinking.

Figure 2 shows a small portion of these three tracings taken from rabbit 609 during a bigeminal pulse following insufflation of benzol. The vagus depressor and cervical sympathetic nerves were severed on both sides at the level of the larynx and respiration was maintained through a tracheal cannula. It is apparent that the apical beat tracing, top tracing and the carotid, middle tracing, show a typical bigeminal pulse. The vena cava tracing at the bottom, however, discloses no sign of a premature beat, each atrial pulse wave is regular and evenly interspaced. The beginning of the arhythmia and return to normal are not included in figure 2, but it can be stated that the bigeminal pulse came on and left abruptly in both the apical beat and in the carotid tracings, while the atrial pulse waves are perfectly normal throughout.

At the suggestion of Doctor Carlson the writer had several electrocardiograms taken of this experimentally produced bigeminal pulse and for these records he is greatly indebted to Doctor Rush for supplying the apparatus and taking the records. Both electrocardiograms shown in figure 2 are from lead two. In this figure the normal pulse of a rabbit is represented by the carotid tracing A and the electrocardiogram B, while the bigeminal

pulse elicited by insufflation of benzol is shown by the carotid tracing C and the electrocardiogram D. The normal pulse is obvious in A and B and the bigeminal in C and D. In addition electrocardiogram D shows the premature systole to be of left ventricular origin.

Observations from inhalation experiments in a previous problem. A bigeminal pulse never followed the first inhalation of an irritating vapor. It always required at least four or five separate stimulations and frequently

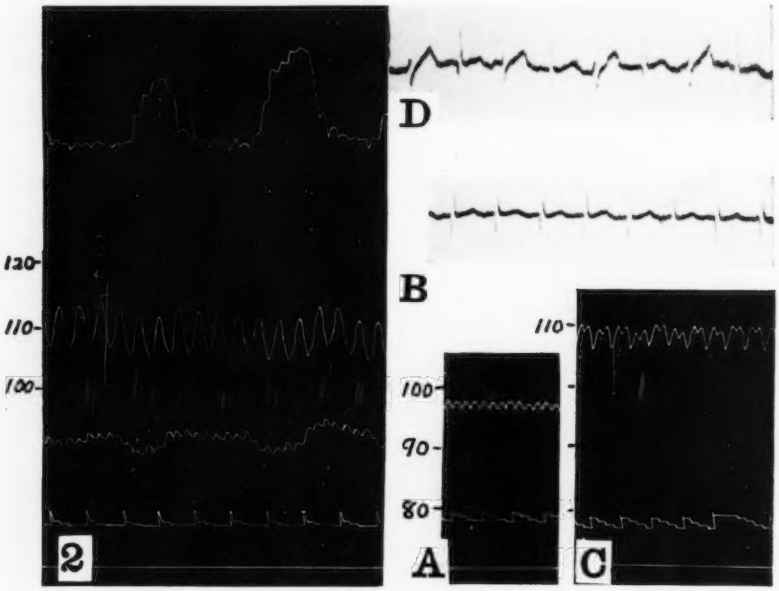

Fig. 2. From a tracheotomized rabbit having vagi, cervical sympathetics and depressors cut. Top row, apex beats; second, carotid blood pressure; third, superior vena cava blood pressure; fourth, time in seconds; fifth, base line, stimulation and arhythmia on.

A and B. Normal carotid pressure tracing and electrocardiogram; C and D, carotid tracing and electrocardiogram of the arythmia.

more to bring it on. It never appeared at the beginning of an inhalation period, but usually came on after the high point in the rise in blood pressure had been reached, sometimes, however, not until inhalation had ceased and blood pressure was falling, and in a few instances not until blood pressure had regained its normal level. The length of time in which the arhythmia persisted varied from 4 to 70 seconds usually lasting from 15 to 30 seconds. A bigeminal pulse followed inhalations of benzol, xylol, formalin, chloroform,

ammonia, acetic acid and mustard oil. Since benzol appeared to be the most favorable inhalant for producing this arhythmia it has been used for almost all of the insufflation experiments.

Observations on the insufflation experiments. In twenty normal tracheotomized rabbits the number of benzol insufflations required to bring on a bigeminal pulse was as follows. It appeared during the first insufflation period in 9 rabbits, in the second in 1, in the third in 2, in the fourth in 2, in the fifth in 2, in the sixth, ninth, fifteenth, and twentieth in 1 each. This shows that in about half of these animals the arhythmia came on with the first insufflation and with three exceptions, six periods of insufflation were sufficient to produce it. Every normal tracheotomized rabbit has yielded an arhythmia at some time following insufflations of benzol.

Several hundred bigeminal pulse records following insufflations of benzol demonstrate variations in time of appearance of the arhythmia. They show, a, that it never follows stimulation immediately as is the case with blood pressure, pulse and respiratory changes; b, that it usually comes on at or near the peak of the blood pressure rise, which is also during the interval of maximum slowing and strengthening of the pulse and the arrest of respiration. Sometimes, however, the arhythmia occurs when these changes are coming on or when the normal is being restored or even after the normal has been regained. An occasional record reveals an arhythmia without pulse changes and with very little blood pressure and respiratory change.

Dogs did not yield the arhythmia from insufflations with anything like the regularity of rabbits. Of three dogs tested, two responded occasionally and one failed entirely. One cat gave negative results.

Relationship of the vagus nerves to the arhythmia. In at least seven rabbits sectioning the vagus, depressor and cervical sympathetic nerves on both sides at the level of the larynx in no way interfered with the mechanism for eliciting the bigeminal pulse from insufflations or faradic and mechanical stimulations of the nasal septum. Many of these carotid pressure tracings show a second bigeminal pulse coming on a short time after the first and other graphs exhibit an apparent lengthening of the bigeminal pulse period. The bigeminal pulse shown in figure 2 was from a rabbit which had its vagus, depressor and cervical sympathetic nerves divided on both sides.

Areas and nerves the stimulation of which have caused a bigeminal pulse. A considerable number of premature systolic arhythmias have been produced in "trigeminal rabbits" (all olfactory fibers cut) from insufflations or inhalations of various irritating vapors. Figure 2 C of a previous paper is such a record. Three out of six "olfactory rabbits" (maxillary and nasociliary nerves cut on both sides) have yielded a bigeminal pulse as a result of insufflations of benzol or ammonia (fig. 1 A). In general this pulse came

on with a few stimulations but in some instances more stimulations were required than in normal and in trigeminal animals. Three olfactory rabbits did not respond to 20 or more insufflations of benzol. It is of especial interest to note that inhalation or insufflation of benzol into a nostril no longer produced a bigeminal pulse in two olfactory rabbits after sectioning the olfactory fibers. All insufflations were made on tracheotomized animals and all olfactory rabbits were autopsied.

The central end of the vagus was stimulated with varying strengths of faradic currents in 14 rabbits. In the majority of these animals the vagus, depressor and cervical sympathetics were cut on both sides at the level of the larynx. A bigeminal pulse was elicited in 10 of these animals and failed in 4. Rabbit 599 required 22 stimulations of the vagus before the bigeminal pulse appeared, after which it came on readily with successive stimulation. In the first arhythmia record stimulation cause a drop in blood pressure and the arhythmia came on during the drop, following an unchanged pulse. Other arhythmias from this and other animals followed a rise in blood pressure.

One to four periods of blowing benzol or ammonia into the trachea or tracheal cannula were sufficient to produce a bigeminal pulse in six rabbits.

The central end of a vagus nerve was stimulated faradically several times in three dogs and one such stimulation was followed by a bigeminal pulse.

A bigeminal pulse followed pulling and uncoiling the small intestine of rabbit 727 preparatory to its inflation.

Since Koblanck and Roeder only obtained this arhythmia after stimulating a small area on the posterior part of the nasal septum in dogs, viz., opposite the middle concha, the writer stimulated various areas on the nasal septum in rabbits electrically and mechanically, with the result that almost any area on the nasal septum from a short distance to the exterior to a point opposite the concha produced a bigeminal pulse. Six rabbits gave the arhythmia readily from one or more stimulations of the septum, while two did not respond to 12 and 14 stimulations. It would appear that the sensitive area on the nasal septum is not so restricted in rabbits as it is in dogs. Also that insufflation of benzol into a nostril is generally a more effective stimulation for bringing on the arhythmia than mechanical or faradic stimulation of the septum.

Areas and nerves the stimulation of which do not cause a bigeminal pulse. In three rabbits the tip of the tongue, the inner lateral wall of the nostril, the inner surface of the larynx, the cornea and various spots on the outer surface of the maxillary and mandibular regions were repeatedly stimulated with varying intensities of faradic currents with negative results insofar as an arhythmia was concerned. As might be expected stimulation of the cornea caused most pronounced vascular changes, but they were not constant. Generally there was a rise in blood pressure and some slowing

and strengthening of the pulse, but sometimes there would be a drop in blood pressure and no pulse changes. Throughout these tests an occasional arhythmia was elicited from insufflations of benzol to determine if the animals were in condition for a sensory impulse to bring on an arhythmia.

In three rabbits a number of inhalations of benzol, ammonia and acetic acid through a tracheal cannula was not followed by an arhythmia, while insufflations of benzol into the trachea and nostril readily produced one.

Opening the abdomen and thorax did not result in an arhythmia, neither did considerable expansion of intact portions of the large and small intestine by injections of warm water.

The central end of the lingual nerve was stimulated faradically 5, 7 and 12 times with currents of varying strength in three rabbits. The results were uniformally a drop in blood pressure (sometimes a drop followed by a rise), a slowed and strengthened pulse, but no sign of a bigeminal pulse. In the above and following tests no records were considered significant unless the animal responded occasionally to an arhythmia from insufflation of benzol.

Several faradic stimulations of the central end of the phrenic nerve in two rabbits did not result in a bigeminal pulse or any especial pulse changes.

The central end of a depressor and a cervical sympathetic nerve were stimulated eight times in rabbit 650 and the central end of a depressor nerve was stimulated four times in rabbit 724 with faradic currents of varying strengths without producing an arhythmia. A typical tracing from a strong stimulation of the central ends of both nerves reveals an 18 mm. drop in blood pressure, a decrease in the rate of the pulse of one wave in 2 seconds and a change in the height of the waves from 1 mm. to 2 mm. The rate of respiration is unchanged but the height of the excursions is reduced by a little over one-half. Both rabbits produced an arhythmia readily from insufflations and central stimulations of the vagus nerve.

Many peripheral faradic stimulations of both vagus nerves in six double vagotomized rabbits and several taken before the second vagus was cut resulted in no arhythmias.

Since the entrance of the sensory fibers of the ulnar nerve is at approximately the same level in the spinal cord as the nuclei of the sympathetics to the heart, the possibility suggested itself that central stimulation of this nerve might produce the arhythmia by a direct spinal cord reflex.

After having obtained a bigeminal pulse once in three different rabbits following insufflation or mechanical stimulation of the nasal septum, the central end of the right ulnar nerve was stimulated 4, 5 and 10 times respectively with faradic currents of varying strengths without any sign of a premature systole or appreciable pulse changes. Typical carotid pressure and thoracic respiratory tracings resemble very closely similar records from

central stimulation of the sciatic nerve (fig. 3). Generally there is a slow rise in blood pressure and a marked inhibition of respiration. Rabbit 727 however gave a fall in blood pressure as often as a rise.

Following a suggestion of Doctor Carlson the sciatic nerve was stimulated centrally in seven rabbits with negative results in so far as this arhythmia was concerned. All were tracheotomized and all yielded the arhythmia readily from insufflations of benzol. It was important in these tests to include respiratory tracings in addition to the carotid tracings because some of the resulting deep respirations frequently conformed very closely to two pulse waves, giving the carotid tracing an appearance in places of a type 2 bigeminal pulse.

Figure 3, the thirteenth faradic stimulation of the central end of the sciatic nerve in rabbit 720, is typical of some 40 records that show no sign of

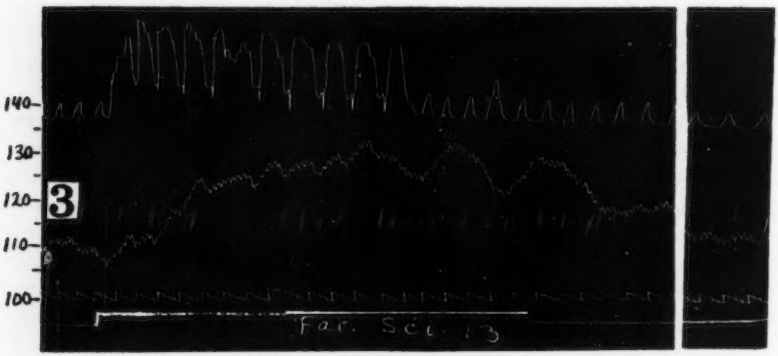

Fig. 3. Thoracic respiratory tracing above and carotid pressure below during weak faradic stimulation of the central end of the sciatic nerve. Inspirations (upstroke) purposely stopped by a bar. No premature systoles shown. Omitted portion is a 21 second interval of the return to normal.

premature systoles. The carotid tracing below demonstrates a rise of 21 mm. during stimulation. The pulse shows no change in rate or strength and the absence of premature systoles is obvious. During stimulation the respiratory tracing above discloses considerable slowing and a marked increase in the depth of the inspirations (upstroke). These excursions which were more than double the height shown were purposely blocked by a bar. Other respiratory tracings reveal an arrest of respiration before the period of deep respiration.

The relation of the carotid sinus to this arhythmia will be considered in the next report.

COMMENT. A bigeminal pulse was elicited: 1, in all normal rabbits and in all "trigeminal rabbits" (olfactory fibers severed) from insufflations; 2,

in one-half of the "olfactory rabbits" (trigeminal nerves to the nasal region severed) from insufflations; 3, in two-thirds of the normal rabbits from mechanical and faradic stimulation of the nasal septum; 4, in five-sevenths of the single and double vagotomized rabbits from faradic stimulation of the central end of the vagus; 5, in six rabbits from blowing benzol or ammonia into the trachea; 6, in one, and only one, rabbit while uncoiling the small intestine. Since many other regions stimulated did not cause this arhythmia, it would appear that the sensory areas of the rabbit which may produce this arhythmia from stimulation are limited chiefly if not entirely to certain upper and lower respiratory areas.

The various inhalation and insufflation tests indicate that it requires either a very powerful single stimulation or a series of weaker stimulations, presumably of the formatio reticularis of the medulla, to call forth this arhythmia. It is also obvious that some animals require many more stimulations than others to evoke an arhythmia.

The arhythmia once obtained in normal rabbits lasts but a short time and reappears only with successive stimulation. The arhythmia can come on after the animal has been subjected to tremendous surgical shock and after many reflexes are lost. It has every appearance of a direct nerve action on the heart.

The writer's experiments with rabbits differ materially from Koblanck and Roeder's concerning the rôle of the vagus. My experiments demonstrate that once having elicited a bigeminal pulse from insufflation, double vagotomy did not prevent stimulations from producing an arhythmia immediately or in any way lessen its effectiveness when it did come on. The only change noticed after double vagotomy was an apparently longer interval of the arhythmia or frequently a second period of bigeminal pulse appearing a short time after the first.

Not only does a bigeminal pulse come on as readily and effectively after sectioning the vagus, depressor and cervical sympathetics on both sides but stimulation of these nerves peripherally has not produced a bigeminal pulse.

According to the writer's experience a bigeminal pulse is produced very easily and regularly in the rabbit from insufflations. Dogs will give it but not with the same regularity and the only cat tried did not respond at all.

SUMMARY AND CONCLUSIONS

A pulsus bigeminus follows insufflations of irritating vapors into the nostrils of tracheotomized rabbits and dogs, from mechanical and faradic stimulations of the nasal septum. It takes place independently of the vagus nerves (confirmation of Kratschmer and Koblanck and Roeder). The arhythmia is also elicited from faradic stimulation of the central end of the vagus in doubly vagotomized rabbits, from insufflation of benzol

or ammonia into the trachea and in one instance it came on while uncoiling the small intestine.

A bigeminal pulse has followed insufflation of benzol, xylol, chloroform, formalin, acetic acid, ammonia and oil of mustard into a nostril.

A bigeminal pulse has not followed—a, faradic or mechanical stimulation of the lateral wall of the nostril, the mucosa of the larynx, the tongue, the cornea, the outer surfaces of the jaws; b, opening the abdomen and thorax or inflation of the large and small intestine; c, faradic stimulation of the central end of the lingual, phrenic, depressor, cervical sympathetic, ulnar and sciatic nerves of the peripheral end of the vagus, depressor and cervical sympathetic nerves.

The usual route of the naso-insufflation stimulation which evokes a bigeminal pulse is obviously over the trigeminal fibers, but under certain preceding experimental conditions the action current probably passes over the olfactory fibers.

Simultaneous apical beat, carotid pressure and superior vena cava tracings of this arhythmia reveal that the right atrium beats rhythmically while the left ventricle is in a premature systolic arhythmia.

In one rabbit, electrocardiograms of several arhythmias demonstrate the premature systole to be of left ventricular origin.

Two forms or types of bigeminal pulse appear in the carotid tracings. They depend on the particular time that the premature contraction appears in the diastolic period. In rare instances the arhythmia may be a trigeminal pulse or a mixture of trigeminal, bigeminal and normal beats.

In carotid pressure tracings the first and succeeding gigantic excursions (type 1) of a bigeminal pulse are very different from the first and succeeding excursions following asphyxia and peripheral stimulation of the vagus.

An arhythmia produced from insufflations usually comes on at the peak of a considerable rise in blood pressure, at the time of maximum slowing and strengthening of the pulse and during the arrest of respiration. It may appear when these changes are coming on, when the normal is being restored or even after the normal has been regained for some time. It sometimed occurs without pulse changes and with very little alteration of blood pressure and respiration.

The experiments reported thus far indicate that the nerve impulse which causes this arhythmia descends the spinal cord and produces the arhythmia either directly or secondarily.

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STUDIES ON SUPRARENAL INSUFFICIENCY

VIII. The Blood Volume of the Rat in Suprarenal Insufficiency, Anaphylactic Shock and Histamine Shock

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Received for publication May 22, 1930

Concentration of the blood during some stage of suprarenal insufficiency has been reported in the case of various animals which die of acute insufficiency within a comparatively short time after double suprarenalectomy. Hartman, MacArthur, Gunn, Hartman and MacDonald (1927) noted an increase in blood solids immediately after or shortly before death in suprarenalectomized cats, and Banting and Gairns (1926) stated that in dogs the total solids increase gradually from the time of operation until death. Zwemer (1927) regarded the symptoms of anhydremia in suprarenalectomized cats as evidence of loss of the liquid constituents of the blood. Gradinescu (1913) reported blood concentration in suprarenal insufficiency in dogs and Swingle (1927) found evidence of marked blood concentration 18 to 20 hours before death in cats. Rogoff and Stewart (1926) observed concentration of the blood in suprarenalectomized dogs toward the end of the survival period, but somewhat in advance of the onset of symptoms in most cases; the change then increased until death. Lucas (1926) observed, however, that the increase in concentration in dogs begins soon after operation, increasing rapidly in dogs of short survival but more gradually on long survival.

Rowntree (1925) made blood volume determinations in ten cases of Addison's disease and found values within normal limits. In one case in which the patient was in a shock-like condition a decreased water content of the blood was found, although the blood and plasma volumes were within normal limits. Because of the condition of the patients further studies were not made.

This paper reports a study of blood volume in normal rats, in rats having cortical suprarenal tissue but no medullary tissue, and in rats having various degrees of cortical suprarenal insufficiency. Blood volume determinations during anaphylactic and histamine shock are also reported.

Methods. A mixed stock of colored varieties of the albino rat was used.

Details regarding care of the animals, the technique of operations, autopsies and histological checks on the experiments, etc., have been described in previous papers in this series. Blood volume determinations were made by the micromethod of Went and Drinker (1929). Adult rats were used for all determinations. The limitations of the vital dye method of determining blood volume were fully realized but comparison of the results obtained by the same method of determination in groups of animals subjected to various conditions should be of significance. Expressing the blood volume as a percentage of body weight is an inaccurate method of presenting data in experiments on suprarenalectomized rats where rapid changes of weight due to the loss of fat are involved. Accordingly, in interpreting the data the cases were considered as individuals, and changes in blood volume following operation were calculated in terms of percentage change of the original plasma and cell volumes determined before operation.

Normal rats and control operation. In twenty-three normal, unoperated rats, eleven males and twelve females, the per cent of plasma of the blood ranged from 51.7 to 60.7, averaging 56.3. The blood volume expressed as per cent of body weight ranged from 6.6 to 9.6, averaging 7.6. This agrees well with the average obtained by Went and Drinker (1929) in a series of nine rats (7.4 per cent), but is somewhat higher than those reported by other investigators who used different methods of determination. No consistent sex differences were noted.

In a series of ten rats, five males and five females, a control blank operation, similar to double suprarenalectomy with the exception of removal of the glands, was done. Blood volume determinations made from 7 to 11 days after operation were within the normal range (plasma per cent 54.1 to 59.8, blood volume 6.9 to 8.9 per cent of body weight).

Autoplastic cortical transplantation. In nineteen suprarenalectomized rats having autoplastic transplants of suprarenal cortical tissue in the abdominal muscles but no medullary tissue as far as could be discovered at autopsy, blood volume determinations made from 41 to 130 days after operation were within the normal range. The plasma per cent was from 50.0 to 60.1, averaging 55.8, and the blood volume was from 6.7 to 9.6 per cent of the body weight, averaging 8.3. There were eight males and eleven females in this series and all were healthy and in good condition. Evidently chromaffin tissue is not essential for the maintenance of a normal blood volume under the ordinary conditions obtaining in a protected animal colony.

Suprarenalectomy. Blood volume determinations were made on forty-four rats, twenty males and twenty-four females, at various times following double suprarenalectomy. In twenty-four cases a blood volume determination was done from 3 to 10 days before operation which could be compared with determinations done after operation. In the others the

determinations done after operation were compared with the normal range and the blood volume was calculated as a percentage of the body weight at the time of the determination and of that at the time of operation. In fourteen cases two determinations were made at different times after operation, thus giving further figures for comparison.

Fourteen rats of this series showed no symptoms of suprarenal insufficiency, remained in good health, and gained considerable weight until they were killed for autopsy from 146 to 187 days after operation. All were found to have gross accessory masses of cortical tissue in or near the suprarenal sites and could be considered biologically normal with respect to cortical function. Blood volume determinations made at various times, from 11 to 176 days after operation, showed no significant changes from the normal. In seven cases two determinations were made, the first from 11 to 36 days and the second from 148 to 176 days after operation. The only differences between these determinations were such increases as would be expected as results of the gain in body weight.

The other rats of the series died of suprarenal insufficiency. In nine which died of acute or subacute insufficiency from 14 to 32 days after operation and in eleven which died of chronic insufficiency from 36 to 98 days after operation no significant changes in blood volume were observed. The blood volume determinations were made at various times before death, from 1 to 7 days in 11 cases and from 11 to 85 days in 9 cases, and in 4 cases two determinations were made a week or so apart. At the time of the determination these rats showed various changes in weight, from a slight gain to a loss of 49 grams; and nine had no symptoms of insufficiency, six had slight symptoms, four had moderate symptoms, and one had severe symptoms.

In eight rats which died of acute or subacute suprarenal insufficiency from 13 to 26 days after operation and in one which died of chronic insufficiency 60 days after operation blood volume determinations made on the day of death (2 cases) or from 1 to 4 days before death (7 cases) showed decreases from the determinations made before operation. The decreases were chiefly in the volume of plasma, being from 10 to 39.6 per cent of its original volume, averaging 21.5 per cent. The volume of cells was decreased only slightly, no more than might be expected in view of the loss of body weight, or was slightly increased (one case). All of these rats had lost weight at the time of the determination, from 28 to 74 grams. Six had severe symptoms of suprarenal insufficiency, two had moderate symptoms, and one had slight symptoms but died on the next day.

In three instances blood volume determinations were made during the terminal stages of suprarenal insufficiency when the rats were comatose and frequently having convulsions. In one case of death from acute insufficiency 8 days after operation the plasma percentage of the blood was much

reduced, being 46.3 per cent. In two cases of death from chronic insufficiency, 95 and 98 days after operation, there was a marked reduction of plasma volume, 22.5 and 21.3 per cent of the original volume, and an increase in cell volume, 12.7 and 9.3 of the original volume.

From these results it may be concluded that in the rat there is a reduction in the total blood volume, due chiefly to a reduction in plasma volume, during the final stages of suprarenal insufficiency or when severe symptoms of insufficiency have appeared. In suprarenalectomized rats which are normal with respect to cortical function because of accessory tissue, and before severe symptoms have appeared in those which are destined to die later of insufficiency the blood volume remains unchanged, except for normal fluctuations.

Anaphylactic shock. Preliminary blood volume determinations were made in ten suprarenalectomized rats having autoplastic cortical transplants but no medullary tissue as far as could be discovered, in four suprarenalectomized rats having gross accessory masses of cortical tissue, and in five normal rats. These rats were then sensitized by intraperitoneal injections of horse serum. Eleven to thirteen days later a test dose of horse serum was given and a blood volume determination was made after symptoms of anaphylactic shock had appeared, or at a suitable time after the test injection (32 to 65 min.). The experiments on the operated rats were done three or four months after operation and all were in good condition and had gained considerable weight. Of the normal rats three had no symptoms and two had slight symptoms. Of the suprarenalectomized rats having transplants or accessory masses of cortical tissue three had no symptoms, seven had slight symptoms and four had severe symptoms of anaphylactic shock, one of these latter dying 177 minutes after the test injection. With one exception, there was a decrease in the plasma volume and a slight decrease or an increase in the cell volume. The changes in cell volume were within the limits of experimental error or no more than might be expected in view of the changes in body weight. The reductions in plasma volume were least in rats having no symptoms of shock (average 13.1 per cent of the original volume, cells 2.2 per cent decrease), greater in those having slight symptoms (average 20.5 per cent, cells 0.5 per cent increase), and marked in those having severe symptoms (average 49.3 per cent, cells 0.7 per cent increase). In the rat which died of anaphylactic shock the reduction in plasma volume, 62 minutes after the test injection of horse serum, was 63.0 per cent of the original volume. It appears, therefore, that anaphylactic shock in the rat is accompanied by a reduction in the volume of the blood plasma, the amount of reduction being correlated with the severity of the shock.

Histamine shock. Suprarenalectomized rats are more susceptible than normal rats to histamine poisoning, this increased susceptibility being

largely due to the lack of medullary tissue (Wyman, 1928). Doses of 10 mgm. of histamine acid phosphate per 100 grams of body weight were given intraperitoneally to seven suprarenalectomized rats having autoplastic cortical transplants, but no demonstrable medullary tissue. One had severe symptoms and six died from 15 to 127 minutes after injection. This dose of histamine causes only slight or moderate symptoms in normal rats. Blood volume determinations made from 10 to 30 minutes after the injections of histamine were compared with determinations made before the injections. The per cent of plasma of the blood fell from an average of about 55 before injection to between 20 and 30 per cent after injection, and there was a marked reduction of the plasma volume, from 36 to 80 per cent of the original volume. The cell volume showed no change or an increase, considerable in some cases, which was probably apparent rather than real. In sixteen normal rats which received various doses of histamine only slight or moderate symptoms were observed. Blood volume determinations were made from 23 to 55 minutes after injection. Eight which received doses of 10 mgm. per 100 grams of body weight showed a reduction of the per cent of plasma of the blood to an average of 44.2, five which received 30 mgm. per 100 grams had plasma percentages averaging 40.3, and three which received 40 mgm. per 100 grams had plasma percentages averaging 35.0. All had corresponding decreases in the plasma volume, and most showed some increase in the cell volume which was probably apparent.

Discussion. A decrease in total blood volume, due chiefly to a reduction in the plasma volume, appears to be a factor in the final stages of cortical suprarenal insufficiency in the rat. Lack of chromaffin tissue alone appears to have no effect upon the blood volume. This is consistent with the results of other investigators who have noted concentration of the blood towards the end of the survival period following suprarenalectomy in other animals. It also confirms the conclusion of Kellaway and Cowell (1922) that the concentration of the blood in cats dying of suprarenal insufficiency is due to cortical defect. Viale and Bruno (1927) found that in dogs the blood concentration is due to loss of whole plasma and suggested that this fact together with the autopsy findings characteristic of acute suprarenal insufficiency indicates that increased vascular permeability is an important factor in suprarenal insufficiency. These findings are consistent with the theory that some influence from the suprarenal cortex prevents the accumulation of toxic products of metabolism in the body by maintaining normal channels of elimination or destruction. Lucas (1926) pointed out that the accumulation of a toxic histamine-like substance due to increased metabolism or to faulty excretion would alter the permeability of the capillaries and bring about concentration of the blood. It is also possible that the diarrhea which is common in suprarenal insufficiency

might contribute to the concentration of the blood. The results reported above also serve to emphasize further the similarity between the symptoms and autopsy findings in acute suprarenal insufficiency in animals, certain conditions observed in some cases of Addison's disease (Rowntree, 1925), and the results of secondary shock. Many authors have spoken of the final stage of suprarenal insufficiency as a shock-like condition.

Belding and Wyman (1926) reported that suprarenalectomized rats four hours after receiving an intraperitoneal injection of diphtheria toxin have 2.75 times as much toxin in the blood as normal rats, and suggested that suprarenal deficiency renders less effective the normal mechanism for the elimination or destruction of the toxin. A possible criticism of this conclusion has been that the greater amount of toxin in the blood of the operated rats is apparent because of a possible concentration of the blood in suprarenalectomized rats. Inasmuch as the reduction of blood volume in the rat is found to occur after the appearance of severe symptoms and the rats in the investigation cited were used during the earlier stages of insufficiency before the appearance of severe symptoms, the criticism mentioned would not be valid.

It has previously been shown that suprarenalectomized rats are more susceptible than normal rats both to anaphylactic shock and to histamine poisoning, and that this is a consequence to the lack of medullary tissue (Wyman, 1929, 1928). The reduction of plasma volume in these two forms of shock is probably a part of the picture of this increased susceptibility. The results suggest that inability to maintain capillary tone and normal permeability, probably due to lack of medullary suprarenal secretion, and resulting in reduction of plasma volume, is the chief factor in increased susceptibility of suprarenalectomized rats to anaphylactic and histamine shock. Kellaway and Cowell (1922) concluded that the altered concentration reaction to small doses of histamine in suprarenalectomized cats is due to medullary defect. If the suprarenal medulla is an important emergency factor in maintaining normal capillary permeability and hence normal blood volume in anaphylactic and histamine shock, the results reported above are further evidence for the suggestion that the suprarenal cortex is concerned in the steady maintenance of certain bodily conditions, while the medulla brings about rapid adjustments in the same direction under emergency conditions (Wyman and Walker, 1929).

SUMMARY

1. In twenty-three normal rats the per cent of plasma of the blood was from 51.7 to 60.7, averaging 56.3 per cent. The blood volume was from 6.6 to 9.6 per cent of the body weight, averaging 7.6 per cent. Following a control blank operation blood volume determinations were within the normal range.

2. In suprarenalectomized rats having autoplastic cortical transplants but no demonstrable chromaffin tissue blood volume determinations were within the normal range.

3. In suprarenalectomized rats which possessed gross accessory cortical tissue and before the appearance of severe symptoms in those which died later of insufficiency the blood volume remained unchanged except for normal fluctuations. After severe symptoms of suprarenal insufficiency had appeared or during the final stages of insufficiency there was a reduction in the blood volume, due chiefly to a reduction in the plasma volume.

4. Anaphylactic shock in sensitized rats was accompanied by a reduction in the volume of the blood plasma, the amount of reduction being correlated with the severity of the shock.

5. Histamine poisoning in rats was accompanied by a reduction in plasma volume, the amount of reduction being roughly correlated with the size of the dose and the severity of the symptoms, being marked in some instances.

6. It is suggested that inability to maintain capillary tone and normal permeability, probably due to lack of medullary suprarenal secretion, and resulting in reduction of plasma volume, is the chief factor in increased susceptibility of suprarenalectomized rats to anaphylactic and histamine shock.

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A BIOCHEMICAL INVESTIGATION INTO THE FUNCTION OF CORPUS LUTEUM

A Study of Blood Sugar and Non-Protein Nitrogen Changes in Rabbits after the Administration of Corpus Luteum

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Received for publication May 26, 1930

There is no need in the present paper of reviewing the excellent physiological experiments that have been done on the function of the corpus luteum. The earliest work in this field (Fraenkel, Loeb, Bouin and Ancel) indicated that the corpus luteum was necessary in the preparation of the endometrium for the implantation and nourishment of the ovum. In the recent and very important investigations of George W. Corner and Willard M. Allen at the University of Rochester, the importance of the corpus luteum for progestational endometrial proliferation and maintenance of early pregnancy has been demonstrated, and most significant contributions in this field have come from their laboratory (1, 2, 3, 4, 5, 6). For the present investigation the ability to procure a highly potent and reliable preparation of corpus luteum such as that described by Corner and Allen (2) has been indispensable.

Since the research to date into the function of corpus luteum has been physiological, morphological and histological in character, it was thought that if some biochemical evidence of the activity of this gland could be found, further light might be shed both upon its function and the mechanism of its action.

Early in 1929, at the suggestion of Dr. George A. Marks, "Corpora Lutea," a Parke, Davis & Company preparation of corpus luteum, was administered to rabbits during pregnancy on the theoretical assumption that an excess of this material would stimulate the endometrium to increased and prolonged activity with consequent added drain on the maternal organism. It was discovered that moderate but definite drops in the fasting blood sugar level occurred in the latter part of pregnancy. Normal and spayed control rabbits did not show any change with continued administration. Later experiments of the same sort with the potent and specific lipoidal extract of Corner and Allen led to the con-

clusion, however, that daily injections of corpus luteum have no marked permanent effect upon the sugar or non-protein nitrogen constituents of the blood of pregnant rabbits.

It was not until the immediate effect of a single injection was followed that any significant results were demonstrated. At first the "Corpora Lutea" preparation was used and, although sugar changes were observed, they were not marked enough to be really conclusive. The use of intraperitoneal injections of the Corner and Allen lipoidal extract confirmed the indications that had already been given by the intravenous injection of the other preparation, but the experiments in which the potent extract was used were so much more striking and conclusive that they only will be reported in the present paper.

The thick lipoidal extract could not, of course, be given intravenously, and the absorption from subcutaneous injection is too slow to be practicable for demonstrating any transitory effects of corpus luteum upon blood constituents. The procedure, therefore, has been to follow the blood sugar, non-protein nitrogen and urea of the blood just before and for several hours after the intraperitoneal injection into a fasting rabbit of from 0.1 to 0.5 cc. of the lipoidal extract.[1] This extract was tested on a spayed doe according to the method of Corner (1) and shown to cause uterine proliferation.

BLOOD CHEMISTRY METHODS. The latest modification of Folin's ferricyanide micro method for blood sugars (7) has been used throughout. For the determination of blood non-protein nitrogen, a micro variation of the regular Folin macro method (8) was devised (by O. W.), making it necessary to draw only 0.2 cc. of blood for each determination. This micro variation was carefully tested out against the Folin macro method on both rabbit and human blood and found to give check values.

During the last three months, however, through the kindness of Prof. Otto Folin, we have been able to benefit by his latest method of precipitating the proteins of the blood without laking the cells (9), and for both non-protein nitrogen and urea have used his as yet unpublished micro procedures. As was shown by Folin, this precipitation of the proteins eliminates the breakdown products of the cell structure and hence, among other things, the so-called "undetermined" non-protein nitrogen constituents of the blood. By using these methods it has been possible to make sugar, urea and total non-protein nitrogen determinations at frequent intervals on one-half a cubic centimeter of blood without exsanguinating the rabbits.

EXPERIMENTAL. The summaries below are based on data derived from

[1] The authors wish to express to Parke, Davis & Company, and particularly to Dr. E. A. Sharp of that Company, their gratitude for the interest and the generous coöperation shown in supplying the valuable extracts used in this investigation.

about three hundred experiments in which intraperitoneal injections of the Corner and Allen lipoidal extract were given and the blood chemistry followed. At certain times the rabbits responded with a marked physical and biochemical reaction; at other times they showed no change at all, either physically or in the sugar or non-protein constituents of the blood. The following is a description of a positive reaction: From ten to twenty minutes after injection the respirations increase markedly in rate and the animal becomes prostrate, with hind legs stretched out. Incontinence often occurs. This condition lasts from one-half hour to two hours. The blood sugar shows rises of from 40 to 300 mgm. above the fasting level, the maximum rise usually coming an hour after injection and returning to normal two to three hours later. The non-protein nitrogen is slower in rising and the time intervals are not so uniform. Usually the rise starts about two hours after the injection, reaches its maximum of 5 to 20 mgm. above the normal from three to five hours after injection and returns to normal eight to ten hours after injection.

The very fact that the animals respond at some times and not at others is suggestive evidence of specificity. To demonstrate conclusively that these changes are the result of the active corpus luteum principle of the lipoidal extract control experiments were run. Rabbits were given intraperitoneal injections of lipoidal extracts of beef muscle and of normal human placenta made by the same process as was the corpus luteum extract. No blood chemistry changes occurred, although the animals were known to be reactive to the corpus luteum at the time the injections were given.

Before considering the mechanism and metabolic significance of these reactions, an attempt was made to correlate their occurrence with the physiological condition of the animal. Brief summaries of the experiments as they were performed are presented.

I. *Normal non-pregnant doe rabbits.* Thirty-eight single injection experiments were carried out upon 18 different animals. Reactions were observed after 11 of these injections, with sugar rises of from 39 to 244 mgm. and total non-protein nitrogen rises of from 4.2 to 21.2 mgm. above the fasting levels. With the remaining injections no reactions or blood chemistry changes occurred. Experiments with pregnant does and with spayed does after treatment with folliculin (see below) make it seem possible that the explanation of these scattered, irregularly occurring reactions is to be found in variations in ovarian activity. Reactions occurred with more regularity in those does which had recently been placed with bucks but not served.

In three of the above animals at another time repeated daily injections were tried over 12- and 22-day periods. Reactions, with marked blood sugar and non-protein nitrogen rises, occurred at irregular intervals. It is at present impossible to say whether the continued administration of

corpus luteum in itself affected the animals or whether the explanation again lies in normal ovarian activity.

II. *Normal does in very early pregnancy.* Seven normal does, shown to be in an unreactive state, were given single injections 34 to 48 hours after being served by bucks. In every case a marked typical reaction followed the injection, with blood sugar rises of from 64.0 to 170 mgm. and non-protein nitrogen rises of from 5.0 to 16.4 mgm. above normal. One unreactive normal doe was tested 25 hours after being served and showed no reaction or blood chemistry change at this time but reacted typically three days later. It was found that a reaction could be predicted with certainty any time from 34 hours to 4 days after serving. In order to determine whether the ovaries were responsible for this reaction the following experiments were performed. Two animals were spayed, one and six hours after being served and the ovaries shown to contain unruptured follicles. They were given injections 37 and 39 hours after castration (43 and 40 hours after being served). No reactions or blood changes occurred at this time or following later injections. Another animal, shown to be unreactive, was served and reacted typically to the corpus luteum injection 43 hours later. Five hours after the reaction it was spayed. The ovaries showed ruptured follicles that contained no demonstrable ova. Twenty-one hours after castration and 69 hours after being served a second injection gave no reaction. This reaction of early pregnancy is apparently dependent upon the presence of the ovaries.

In two animals the effect of hysterectomy without oöphorectomy was tried. Following hysterectomy both animals were shown to be unreactive. Forty and 48 hours after being served, typical reactions followed the injection of corpus luteum. The uterus is apparently not necessary for the early pregnancy reaction.

III. *Normal does throughout pregnancy.* Thirty single injections into 9 different animals from the fifth day of pregnancy to delivery resulted in 10 typical reactions with sugar rises of from 51 to 200 mgm. and non-protein nitrogen rises of from 5.0 to 9.2 mgm. above the normal. No correlation could be made between these reactions and the stage of pregnancy or the known physiological changes of the organism.

IV. *Lactating does.* Fifteen injections of corpus luteum were given to 9 different animals 6 days or less after delivery. Of that number all but two gave typical reactions with blood sugar rises of from 77 to 258 mgm. and non-protein nitrogen rises of from 6.5 to 22.9 mgm. above the fasting levels. The two exceptions were rabbits that received the injections at 14 and 21 hours, respectively, after delivery.

The results later in lactation were less consistent. Seventeen injections were given to 4 different animals from the seventh to the twenty-first day after delivery. Nine of these injections resulted in typical reactions, with

blood sugar rises of from 39.0 to 294 mgm. and non-protein nitrogen rises of from 3 to 9.5 mgm. above the fasting levels. As far as could be determined the reactions were not in any way dependent upon whether or not the animal was nursing its young or upon the time interval following delivery.

In the attempt to discover whether or not, as in early pregnancy, the reactions after delivery depended upon the presence of the ovaries the following experiments were carried out. One of the above animals, shown to be reactive on the second day after delivery, was spayed. The ovaries contained no ruptured follicles. On the next day, four days after delivery, an injection resulted in a typical reaction with blood sugar and non-protein nitrogen rises. On the sixth and eleventh days after delivery, however, three and eight days after castration, no reactions were obtained. A second animal, shown to be reactive 30 hours after delivery, was operated upon and all uterine as well as ovarian tissue removed. Forty-five hours and also four days later, on the third and sixth days after delivery, typical reactions to the injections occurred. Another animal was spayed on the 25th day of pregnancy. Premature labor occurred on the 27th day. One day later a corpus luteum injection resulted in a typical reaction. The reactions after delivery, then, unlike those in early pregnancy, can apparently occur when the ovaries are absent. The removal of the uterus, furthermore, seems to have no effect upon the occurrence of the reaction.

V. *Spayed does.* Twenty-eight single injections were given to 8 doe rabbits that had previously been spayed. With three exceptions no reactions occurred. One of these exceptions was an animal that reacted 17 days after castration. The other two reactions occurred in another animal on the fourth and tenth days after it was spayed. At the time of operation this second animal showed extreme congestion or uterus, vagina and ovaries. It had been receiving large doses of the lipoidal extract subcutaneously for three months prior to the operation.

Repeated intraperitoneal injections were given to two spayed does. One received 12 injections over a period of 27 days. It gave no sign of any reaction except on two occasions, when the sugar rises were only 20 and 29 mgm. respectively. The second animal received 19 injections over a period of 30 days and showed no reaction except once, when the sugar rise was only 22 mgm.

From the above it would seem safe to assume that ordinarily a spayed doe shows no physical reaction or blood sugar or non-protein nitrogen rise after the injection of corpus luteum.

VI. *Bucks.* Twenty-four injections were given to 8 buck rabbits. Only four reactions resulted, three of these being in the same buck, no. 40, and the other in a young buck. Another buck that had been shown to be unreactive on four different occasions gave typical reactions with marked

sugar and non-protein nitrogen rises on the second, sixth and eighth days after castration. On the 14th day it still showed a slight sugar rise, but four more injections during the next 20 days were entirely negative. It would appear from this that castration had a temporary effect upon the animal, making it reactive to corpus luteum. This does not, however, explain the occasional reactions of buck 40, and although it may be said that in general normal buck rabbits give no physical reaction or blood changes following the intraperitoneal injection of corpus luteum, these few unexplained exceptions made it impossible to predict with certainty that no reaction will occur.

VII. *Effect of folliculin upon the reactiveness of bucks, spayed does and immature rabbits.* Considering the fact that in most of these reactions the all-or-none law seems to apply and particularly considering the consistent finding of a reaction after serving a doe when ovulation is known to take place, it occurred to the investigators that a change in reactiveness so strikingly sudden might be due to the release of some hormone rather than to any more gradual physiological changes involved. In these post-ovulatory reactions the active principle of the follicle fluid was naturally considered.

In the experiments with folliculin the following sources of material have been used: 1, urine of women 6 to 9 months pregnant; 2, ether extracts of the urine of pregnant women; 3, olive oil extracts of the urine of pregnant women (10); 4, lipoidal extracts of human placentas prepared by hot alcohol extraction and subsequent removal of phospholipids as described by Corner and Allen (2). The material was tested for its estrus-producing power on spayed white rats according to the method of Allen and Doisy (11), three injections being given at four-hour intervals, the amount producing a full estrus smear at forty-eight hours from the first injection being considered one unit. The approximate amount of folliculin administered varied from ten to twenty-five rat units.

Three normal buck rabbits, shown to be non-reactive to the corpus luteum, were given either subcutaneous or intraperitoneal injections of folliculin preparations whose estrus-producing properties had been demonstrated on spayed rats. The intraperitoneal injection of corpus luteum was then given and resulted in typical reactions with blood sugar rises of 116 to 155 mgm. and non-protein nitrogen rises of from 7.5 to 13.5 mgm. above the fasting levels. In two of these rabbits an ether extract of pregnant urine was given intraperitoneally and the corpus luteum injection given the next day. In the third, 40 cc. of pregnant urine were given subcutaneously daily for three days before the intraperitoneal corpus luteum injection was given. As has been stated, a typical reaction occurred at this time, but the same animal 9 days later gave no reaction or blood changes.

Seventeen experiments were carried out in which corpus luteum, following

the administration of folliculin, was given to five spayed does, previously shown to be non-reactive. A positive result was obtained in every instance, the blood sugar rises ranging from 20 to 92 mgm. and the non-protein nitrogens from 5.4 to 9.5 mgm. above the fasting levels. These rises are less than those reported in the previous experiments, probably for the following reasons: 1, less folliculin than would be calculated to be necessary on the basis of the rabbit-rat weight ratio was used; 2, subcutaneous absorption was slower than intraperitoneal; 3, oily and lipoidal extracts are very much more slowly absorbed than aqueous preparations; 4, the optimum time interval between the giving of folliculin and the experiment has not yet been determined.

Four young doe rabbits, less than four months old, were tested with the lipoidal corpus luteum and three found unreactive. These three were then given eight 1 cc. injections of an ether extract (1 cc. equal to approximately

TABLE 1

Maximum non-protein nitrogen and urea rises following the intraperitoneal injection of corpus luteum into rabbits

RABBIT	CONDITION	N.P.N. AND UREA RISES 5 TO 6 HOURS AFTER INJECTION		
		N.P.N. old	N.P.N. new	Urea new
		mgm. per 100 cc.	*mgm. per 100 cc.*	*mgm. per 100 cc.*
No. 22	Buck after folliculin	7.5	7.3	5.7
No. 33	Doe after spaying	9.5	8.8	8.5
No. 65	Doe 2 days pregnant	13.1	6.6	4.6
No. 65	Doe 4 days pregnant	10.0	7.1	7.3
No. 49	Spayed doe after folliculin	7.0	7.8	6.6
No. 49	Spayed doe after folliculin	7.5	9.6	9.5

1 unit) of urine from pregnant women over a period of three days and tested again on the fourth day. The typical physical changes and blood sugar rises of 30, 42 and 60 mgm., respectively, were obtained.

The fact that different sources of folliculin were used in the above experiments indicates that an active specific hormone produced the consistent results. As a further check, one spayed doe rabbit was given subcutaneously urine from a woman who had passed the menopause. This urine gave a negative rat test and failed to make the rabbit reactive to the corpus luteum.

It may be concluded from these experiments with folliculin that any unreactive animal may be made to respond typically to corpus luteum by first bringing it under the influence of the estrus hormone.

MECHANISM OF THE REACTION. Before discussing the results given above, a brief report will be given of some work done in an attempt to derive information about the mechanism of these blood chemistry changes.

Table 1 illustrates the results of experiments done to determine the relative rises in non-protein nitrogen and urea of the blood. The non-protein nitrogens were determined both by the micro variation of the old Folin method and by the new micro Folin method of precipitation of proteins without laking the cells. The ureas were done by this new method only. An examination of the table shows that the non-protein nitrogen rise is almost entirely accounted for by the rise in urea. The differences between the results of using the two different non-protein nitrogen methods must be due to changes in cell volume. Red blood cell counts were followed in a number of experiments and it was found that they fluctuated considerably, irrespective of any sugar or non-protein nitrogen rises. This merely emphasizes the greater accuracy of the new Folin methods for non-protein nitrogen constituents, particularly on rabbits' blood.

Both liver and kidneys were considered as possible factors in the non-protein nitrogen changes. In order to discover if poisoning the kidney gave curves comparable to those given by corpus luteum injections, an intraperitoneal injection of 400 mgm. of lithium urate was given to a buck rabbit weighing two kilograms. The blood urea showed no change at all for five hours. At the sixth hour it had started up and at the 15th hour was 12 mgm. above normal. At the 22nd hour it was still 5.4 mgm. above the fasting level. There was a slow sugar rise of 80 mgm., which reached its maximum four hours after the injection and had returned to normal eleven hours later. These rises in sugar and urea occurred later and were much more prolonged than those from corpus luteum. In frequent experiments in which the non-protein nitrogens were followed for as long as 10 hours after the corpus luteum injection, it was always found that they were nearly back to normal by this time. In the case of lithium urate, however, the urea was still 3 mgm. above normal three days after the experiment. This prolonged effect of lithium urate gives suggestive but not conclusive evidence that the urea rise after corpus luteum is not simply the result of poisoning the kidneys.

Attempts to follow the urinary excretion of urea after corpus luteum reactions were unsatisfactory because of the difficulty in catheterizing rabbits. Tests for urinary albumin (Esbach) and examination for casts also gave inconclusive evidence of renal upset, for domestic rabbits show frequent variations in these products under ordinary laboratory conditions. No definite statement can be made, therefore, as to whether or not the kidneys are affected in these reactions.

At the suggestion of Dr. Fuller Albright, epinephrine in 1 cc. doses of the 1 to 1000 solution was given intraperitoneally. The results revealed a marked similarity in the effect of epinephrine and corpus luteum upon the sugars and non-protein nitrogen constituents of the blood of rabbits. As

with the corpus luteum injections, the maximum rises in sugars came one hour after injection and had returned to normal three hours later. The maximum rises in non-protein nitrogens came four to six hours after injection and had started back towards normal by eight hours after injection. All of the rise in non-protein nitrogen was accounted for by rise in urea. This evidence seems to indicate either that the lipoidal corpus luteum may have a chemical relationship to epinephrine or that it specifically stimulates the sympathetic nervous system under certain conditions. Possibly it has a direct effect upon the liver, kidneys or adrenals through the blood stream. The fact that for the first time, apparently, epinephrine has been found to cause rises in the blood urea of rabbits is in itself worthy of note, and suggests that similar exaggerated catabolic and possibly toxic effects may be produced by both of these substances. These hypotheses are of particular importance in considering the relation of corpus luteum to the problems of pregnancy.

Discussion. The fact that such marked blood chemistry changes were found to occur at certain times in rabbits following the intraperitoneal injection of corpus luteum, and that these reactions consistently occurred in the animals that had been given folliculin, at once suggests that certain metabolic changes depend upon a balance between these two endocrines. This biochemical evidence of the relation of these two hormones is in accord with the recent physiological findings of Hisaw and Leonard (12) and of Willard M. Allen (6), namely, that the progestational proliferation of the endometrium produced by means of corpus luteum depends upon the presence of folliculin. Allen found that in immature rabbits only a very small percentage responded to progestin treatment, and that in those that did not respond, progestational proliferation could be produced by first bringing the uterus under the influence of oestrin. Our experiments on the immature rabbits reported above are in exact accord with these findings of Allen.

The regularity with which rabbits that had recently been served responded to corpus luteum injections indicates an association with increased ovarian activity, since rabbits are known to ovulate after copulation. It is logical to assume that the ovarian activity at this stage results in increased folliculin secretion. The irregular reactions of normal and pregnant rabbits may be the result of variations in ovarian activity with consequent variations in folliculin secretion. Since, however, reactions have been obtained in lactating does following castration, it would seem that ovarian activity is not the only factor to be considered. It is not inconceivable that the sex principle of the anterior pituitary is also involved at times, and further work with this possibility in mind may serve to explain some of the reactions in which the ovaries could not have been involved. It may be mentioned in this connection that three very young

rabbits still nursing, showed the typical physical picture when given 0.1 cc. of corpus luteum intraperitoneally, whereas these same rabbits did not react after they had been weaned. The anterior pituitary, then, may be involved in the reactions both of lactating does and of their nursing litters. These suggestions concerning the rôle of the anterior pituitary are purely hypothetical and are presented merely to show that the influence of this gland has not been ruled out in the present investigation.

SUMMARY

Intraperitoneal injections of the Corner and Allen lipoidal extract of corpus luteum were given to rabbits (normal non-pregnant, pregnant, lactating and spayed does, and normal bucks) and resulted at certain times in marked physical reactions accompanied by rises in the sugar and non-protein nitrogen constituents of the blood. Control experiments demonstrated that these reactions were specific for the active corpus luteum principle of the extracts used.

In the majority of cases, non-pregnant doe rabbits, pregnant rabbits after the fourth day of gestation and lactating rabbits after the sixth day postpartum gave no physical or biochemical evidence of responding to the injections. Occasionally, however, marked typical reactions occurred. No correlation could be made between these irregularly occurring reactions and the known physiological state of the animals.

It may be predicted with certainty that normal doe rabbits will react typically to injection any time from 34 hours to 4 days after copulation. The ovaries at this time show ruptured follicles. If the animal is spayed after being served the reaction does not follow injection.

From 24 hours to 6 days after delivery normal does give positive reactions to the injection of corpus luteum. The post-delivery ovaries show no evidence of ruptured follicles and removal of them does not prevent the reaction.

Removal of the uterus has no effect upon the response to corpus luteum either in early pregnancy or after delivery.

Spayed does and normal bucks (with a few exceptions) do not show any physical or biochemical changes following the intraperitoneal injection of corpus luteum, but if these unreactive animals are first brought under the influence of folliculin, the corpus luteum injection is always followed by a positive reaction. This finding indicates that the consistent reactions of does after copulation and the occasional reactions of non-pregnant and pregnant does are the result of the normal production of folliculin within the organism. This biochemical evidence confirms the recent physiological findings of Hisaw and of Willard M. Allen of an interrelationship between corpus luteum and the estrus hormone. The behavior of lactating

does, however, cannot be explained upon this basis and the possible influence of the sex principle of the anterior pituitary has not been eliminated.

The rise in non-protein nitrogen is almost entirely accounted for by rise in urea. Although this points to renal upset, the direct poisoning of the kidneys by lithium urate does not give comparable sugar and urea curves. The intraperitoneal injection of epinephrine, on the other hand, results in both non-protein nitrogen and sugar rises practically identical, both in magnitude and time relations, to those following a positive corpus luteum reaction. This finding suggests that the corpus luteum under certain physiological conditions either stimulates the sympathetic nervous system or else has a direct effect upon the organism similar to that of the adrenal medulla. Whether or not the urea rise is a secondary result and possibly due to kidney damage has not yet been ascertained.

It seems possible that the biochemical changes observed following the injection of single large doses of corpus luteum extract may be an exaggeration of a normal catabolic action of this gland. This effect would be entirely in keeping with the importance of the corpus luteum for the implantation and nourishment of the ovum.

To Prof. Otto Folin for his constant and valuable assistance and advice and to Dr. George W. Corner and Dr. Cecil K. Drinker for their helpful interest the writers wish to express their deep appreciation.

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SEX HORMONES IN THE BLOOD SERUM OF MARES

II. The Sera of Mares from the 222nd Day of Pregnancy to the First Heat Period Post-partum

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Received for publication May 24, 1930

In a previous publication (Cole and Hart, 1930) data were presented on the hormones in the blood of gravid mares affecting the genital system of the immature rat, with particular reference to the sex-maturing hormone of the anterior hypophysis. The presence of this hormone makes possible a highly accurate biological test for the early diagnosis of pregnancy in this species. It was also demonstrated that there is a variation in the concentration of this hormone at different stages of gestation, which was studied from the time of conception to the 222nd day of pregnancy. A close correlation was observed between the time of greatest concentration and the time of implantation. Data were also obtained on the presence of folliculin in the serum.

Our study of the blood serum in mares has been continued to the end of gestation, through parturition, and to the first heat period following, which usually occurs about the ninth day postpartum. The methods used in this study were fully described in the first publication.

Experimental. *Vaginal changes induced in the experimental animals.* The changes in the vagina characterized by the presence of the smear of oestrum can be demonstrated in immature rats following the injection of gravid serum in sufficient quantities into these animals during the greater part but not all of the gestation period. The absence of any genital response was previously noted for the period from 37 to 42 days after conception. There is also some variation in the time at which the reaction of the serum becomes negative preceding parturition. The length of pregnancy and the reaction of the serum before and after parturition on four mares is recorded in table 1.

It may be seen that the serum is negative in each instance when obtained at one day postpartum.

The data indicate that from about the 150th day to approximately the end of gestation, the dose of serum required to produce reactions in the vagina of the rat is reasonably constant. Doses of 15 cc. or less were

negative in some cases and positive in others, and this dose is therefore on the borderline for eliciting the reaction. Doses of 25 cc. or more were uniformly positive up to approximately the end of the gestation period. It is thus shown that the amount of serum required to produce this reaction remains quite uniform from about the 150th day to approximately the end of pregnancy. The period of pregnancy in which the serum will produce a positive vaginal reaction is shown in figure 1. However, one must bear in mind that a fraction of a cubic centimeter will produce the reaction in the early stages whereas in the later stages about 25 cc. are necessary.

Changes in the uteri of experimental animals. It may be seen from figure 1 that the curve of uterine weights follows fairly closely that of the curve of the ovarian weights. The one notable exception is that the uterine weights do not diminish until the ovarian weights are approaching the weights of

TABLE 1

Vaginal and uterine reactions of rats receiving serum obtained from mares before and after parturition

NUMBER OF MARE	LENGTH OF PREGNANCY	DAYS ANTEPARTUM	REACTION	DAYS POSTPARTUM	REACTION
	days				
2	349	4	Positive	1	Negative
5	345	22	Positive	1	Negative
		6	Negative		
7	351	14	Positive	1	Negative
		7	Negative		
28	339	2	Positive	1	Negative

the ovaries of control animals. This indicates that a small ovarian reaction will produce a maximum reaction in the uterus. We have previously noted that in the earlier days of pregnancy (37 to 42 days) even though the ovarian reaction was weak, there was a maximum reaction in the uterus. From the 150th day until the end of pregnancy the uterine weights are uniformly greater than the weights of control uteri. Also in practically every instance, the uteri from injected animals contained a small but macroscopically perceptible amount of fluid at the tips of the uterine horns. Inasmuch as there is evidence of ovarian inhibition in the experimental animals, this slight reaction must have been the result of some factor in the serum injected. We are not able to explain why a distinct vaginal reaction and a very weak uterine reaction occurs with serum from mares late in pregnancy. Considering the gestation period as a whole, a slight uterine reaction was found to be regularly associated with a distinct reaction

in the vagina and a negative reaction in the ovaries. A marked uterine reaction has always been associated in our experience with a similar vaginal reaction and an increased development of the ovary, as represented at least by the enlargement of a single follicle.

Changes in the ovaries of experimental animals. In the former paper it was shown that the ovarian reaction could not be elicited after about the 175th day of pregnancy. We were surprised after the accumulation of more data to find that the ovaries of the experimental animals were actually smaller than those of the controls when injected with serum obtained from

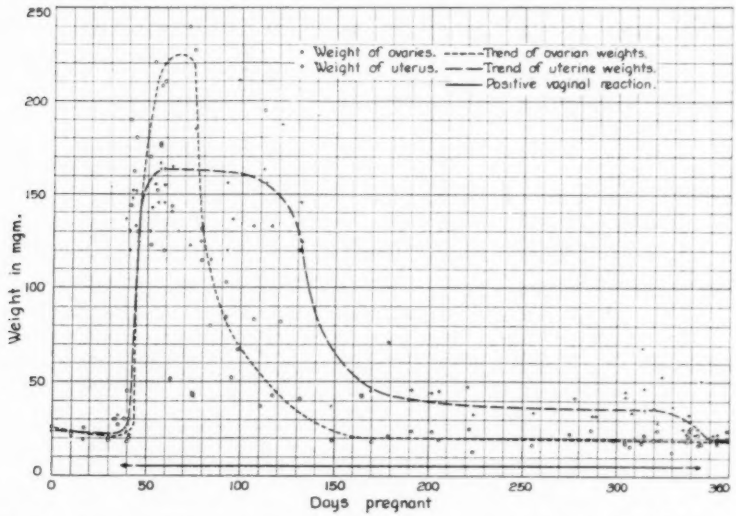

Fig. 1. Average weights of ovaries and uteri of rats injected with serum taken from mares at various stages of pregnancy. Weights of ovaries include oviducts and bursae. Period in which serum produced a positive vaginal reaction indicated by unbroken line.

about the 150th day to the end of pregnancy, even though a distinct reaction of oestrum was produced in the vagina. A comparison of the weights of ovaries of injected animals and their littermate controls is given in table 2. It is evident from this table that the sera of mares late in pregnancy had an inhibiting action upon the development of the ovaries.

The data were subjected to statistical study to ascertain the standard errors of the mean weights of the ovaries of the injected and the control groups. The average weight of the control ovaries was 13.7 ± 0.68 mgm., while that of the injected animals was 9.4 ± 0.42 mgm. It was found that the difference in the weight of the ovaries of the injected as compared to

TABLE 2

Inhibiting effect of blood serum in late stages of pregnancy on ovaries of immature rats. Oviducts and bursae removed before weighing

INJECTED RATS							LITTERMATE CONTROLS		
Number of mare	Days pregnant	Number of rat	Age of rat	Amount of serum injected	Body weight	Weight of ovaries	Number of rat	Body weight	Weight of ovaries
			days	*cc.*	*grams*	*mgm.*		*grams*	*mgm.*
7	266	W 1010	32	55	83	10.0	B 1012	89	20.0
		W 1009		55	89	11.5	B 1013	89	19.0
		B 1011		110	88	15.0			
7	277	W 1053	25	30	53	11.5	W 1054	55	17.5
		BH 1049		30	51	8.5	W 1052	53	15.0
		BH 1050		30	53	10.0			
		W 1053		30	53	11.5			
7	277	B 1057	25	30	54	10.5	B 1055	64	15.0
							B 1056	56	14.0
7	277	B 1059	25	30	52	11.0	W 1058	53	11.0
		BH 1060		30	49	10.5	BH 1061	57	16.5
9	297	W 1315	26	35	57	13.2	B 1317	52	16.8
		B 1316		21	48	12.4			
5	305	W 1299	25	30	51	7.0	GH 1298	46	8.0
							W 1301	50	13.0
9	305	B 1326	25	30	46	6.4	B 1328	43	6.8
		G 1327		30	42	6.8	B 1329	47	11.0
9	305	B 1354	27	50	52	9.2	G 1357	58	17.0
		G 1355		50	52	10.6	G 1358	58	16.2
		G 1356		50	59	10.4	G 1359	52	13.0
5	313	B 1389	26	50	46	7.6	W 1372	46	12.0
		B 1370		50	43	7.8			
2	315	B 1256	25	30	46	9.8	W 1260	46	15.4
		B 1257		30	49	9.8	W 1248	60	11.5
		W 1258		20	48	8.0			
		W 1259		15	48	10.6			
2	315	G 1282	25	25	56	11.0	B 1285	54	12.4
		G 1284		20	56	9.2	G 1287	57	16.0
2	320	G 1271	25	30	49	8.2	G 1273	49	15.4
		G 1272		15	51	9.2	G 1274	47	10.0

TABLE 2—*Concluded*

INJECTED RATS							LITTERMATE CONTROLS		
Number or mare	Days pregnant	Number of rat	Age of rat	Amount of serum injected	Body weight	Weight of ovaries	Number of rat	Body weight	Weight of ovaries
			days	*cc.*	*grams*	*mgm.*		*grams*	*mgm.*
2	324	G 1324	25	30	54	9.0	B 1328	43	6.8
		B 1325		30	51	7.6	B 1329	47	11.0
2	345	BH 1380	25	30	41	4.0	GH 1385	40	7.6
		BH 1381		30	41	3.8			
		GH 1383		30	41	4.0			
9	317	B 1360	30	80	74	10.6	B 1363	69	16.4
		B 1361		80	64	12.6	B 1364	71	15.8
		B 1362		80	68	8.8	B 1365	69	18.0
Average					54.39	9.4		55.8	13.6

that of the control animals was five times the standard error of the difference between the means and therefore highly significant. The difference between the means was 4.3 mgm. and the standard error of the difference between the means was 0.8 mgm.

By referring to the table it may be seen that the difference is much greater when larger amounts were given. In the instances in which more than 30 cc. were given the injections were extended over a longer period, thus giving additional time for the ovaries of the controls to continue normal growth.

A comparison of the ovaries of control and injected rats is shown in figure 2. The only difference between the two appears in the greater follicle development of the control ovary, particularly as regards the number of large follicles.

We have made no attempt to determine what factor, or factors, are responsible for this inhibiting effect upon the ovary. Long and Evans (1921) showed that the growth-promoting hormone of the anterior hypophysis inhibited the development of the ovary. Many investigators have shown that the corpus luteum contains a factor which inhibits the physiological activity of the ovary. As this paper was being prepared, it was noted that Leonard, Meyer and Hisaw (1930) reported on oestrin having an inhibiting effect upon the development of the ovary of the immature rat. The ovaries of their injected animals were on an average $33\frac{1}{3}$ per cent lighter than their littermate controls. In their work immature rats 20 to 25 days old were given daily injections of 2 to 4 rat units of oil soluble oestrin for 20 to 27 consecutive days. Nibler and Turner (1929) made an

alcoholic extraction of oil containing the ovarian hormone from cows' urine. They found an apparent increase in the number of rat units present in a definite volume of the oil as a result of the extraction. It is possible that Leonard, Meyer and Hisaw were injecting not only oestrin but also an additional factor present in the oil. In our animals injections for only three consecutive days were necessary to demonstrate inhibition at 96 hours

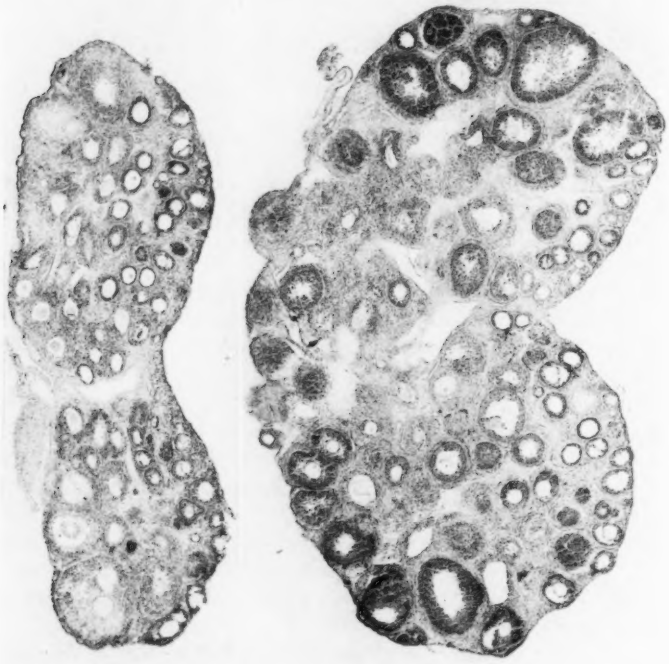

Fig. 2. Cross sections of ovaries of injected rat, G 1357, (left) and its litter mate control, G1356 (right). ×33.

from the time of the first injection. We have demonstrated the presence of the inhibiting factor from the 150th day to the end of pregnancy. We demonstrated the presence of oestrin in serum taken on the 222nd and 256th days of pregnancy with spayed rats and it may be present throughout this period. However, we are not convinced that oestrin is causing the inhibition.

The ovaries of twenty rats receiving serum from non-pregnant mares

did not show any evidence of this inhibiting effect which gives further evidence that this is a reaction characteristic of a definite stage of pregnancy.

SUMMARY

Serum was collected from mares between the 222nd day of pregnancy and the first heat period following parturition to study its effect upon the genital system of immature rats. The serum was found to produce an effect upon the genital system until about the time of parturition. A positive vaginal reaction, a weak uterine reaction, and an inhibiting effect upon the ovaries were regularly observed. No reaction was observed from serum taken after parturition had occurred.

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THE QUESTION OF PLURISEGMENTAL INNERVATION OF CERTAIN MUSCLES IN THE FROG[1]

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Received for publication June 9, 1930

The question of plurisegmental innervation of skeletal muscle invites morphological study not only because this type of innervation frequently forms the basis for the interpretation of physiological findings, but also because of its relation to the plurisegmental origin of muscle fibers and to muscle changes following injury of cord segments or nerve roots.

In the spring of 1928 this study was begun, to determine the percentage of distribution of plurisegmentally innervated muscle fibers, already noted by Hines (1927) in the M. sartorius of the frog. Later in the same year Lawentjew (1928) published his report on the question of plurisegmental innervation of the M. gastrocnemius and M. sartorius in Rana esculentia and temporaria. With the Bielschowsky technique he demonstrated that the contributing nerve roots supply distinct regions of the frog's gastrocnemius with only a small area of overlap. Within this area of overlap no plurisegmentally innervated muscle fibers were seen. On the other hand, there were no such distinct regions in the M. sartorius; nerve fibers from adjacent spinal segments frequently ran in mixed bundles. Plurisegmentally innervated fibers frequently occurred. These are the only definite data concerning the relation of nerve roots to muscle fibers in the frog, and Lawentjew used Rana esculentia and temporaria. On the other hand there is no adequate knowledge of the innervation of the M. gastrocnemius and the M. sartorius of Rana pipiens, the form primarily used in physiological studies in plurisegmental innervation. This study was continued then with the use of a technique, adequate to state the anatomical relation of segmental origin of innervation to the muscle fibers of the sartorius and the gastrocnemius, the distribution within these muscles of the nerve fibers from each contributing nerve trunk, and the variation that might be expected in the trunks supplying nerve fibers to these muscles.

METHODS AND TECHNIQUE. In the following experiments small frogs of the species *Rana pipiens* were used. The lumbo-sacral plexus in these frogs is composed mainly of the ventral divisions of three spinal nerves,

[1] This work was carried on during the holding of a grant from the Henry Strong Denison Medical Foundation.

N VIII, N IX, and N X according to the nomenclature used in Ecker-Wiedershiem's *Anatomie des Frosches*. These correspond to N VII, N VIII, and N IX in the nomenclature used by many experimentalists who name the hypoglossal nerve "N I" as there is no spinal nerve anterior to it in the frog. The nomenclature of Ecker-Wiedersheim will be used in the following description.

In order to study the distribution of the motor nerve fibers in these nerve trunks within the gastrocnemius and sartorius muscles a series of operations was performed in which (1) N VIII, or (2) N IX, or (3) N X was cut on one side of the animal. In another series the anterior root of (1) N IX, or (2) N X was cut within the vertebral canal and in a third series two of the three nerves were cut. A sufficient interval was allowed for degeneration of the motor endings of the cut fibers. This varied from 9 to 14 days according to the season of the year, a longer degeneration time being required in winter. At the end of this period, the muscles were prepared by the methylene blue technique developed by Hines for the frog, i.e., 400 cc. of methylene blue (1 part methylene blue to 5000 parts 0.88 per cent NaCl solution) were injected by gravity through a cannula inserted into the conus arteriosus. The skin was removed from the legs and the belly of the gastrocnemius on each side slit longitudinally. The muscle was pinned open so that its innermost fibers would be well aerated. The *M. sartorius* is so thin that removal of the covering fascia gives good aeration throughout. After two hours of exposure the preparations were fixed by injecting through the same cannula 250 cc. of 5 per cent ammonium molybdate. They were allowed to stand from 5 to 6 hours or, in cool weather, overnight. The muscles were then removed, washed with distilled water for 5 minutes and mounted in glycerine. With this technique degenerated motor end-plates could be readily distinguished from normal motor end-plates.

EXPERIMENTAL FINDING. *Innervation of the fibers of the gastrocnemius.* When either N IX or N X is cut, degenerated fibers can be readily demonstrated in the gastrocnemius. However, in only one case out of seven in which the gastrocnemius was studied after section of N VIII was there any degeneration seen.

On cutting NX the usual position of the degenerated fibers is at the periphery of the upper pole, generally more toward one side than the other. In about one-third of the cases, however, there were also a few degenerated fibers at the lower pole.

Cutting N IX results in the degeneration of all motor endings in the muscle except those in the position corresponding to the distribution of N X, namely, those supplying a small region at the periphery of the upper pole. In a few cases some normal endings were found at the lower pole.

The great majority of nerve fibers in the gastrocnemius, then, are derived from N IX and are distributed throughout the lower pole, middle, and part

of the upper pole of the muscle, while N X supplies a small area at the periphery of the upper pole.

In the one case in which degeneration resulted from cutting N VIII, two small degenerated bundles were found at the lower pole of the muscle.

There are, therefore, in the gastrocnemius only two small regions where it would be at all possible for plurisegmental innervation to exist,—at the extreme upper pole and rarely, at the extreme lower pole. But the shortness of the muscle fibers themselves within those regions makes the chance of finding bisegmental innervation exceedingly improbable.

A detailed study was made of 28 gastrocnemii from frogs in which either N IX or N X had been cut to determine whether or not fibers from different spinal segments ever terminated on the same muscle fiber. In all these preparations the degeneration was sufficiently advanced so that normal motor endings could be readily distinguished from degenerated motor-endings.

In 25 of the 28 gastrocnemii no instance of plurisegmental innervation was discovered. In two muscles one fiber was found bearing a degenerated and a non-degenerated ending while in a third muscle there were two fibers each with a degenerated and a non-degenerated ending.

From these results it is evident that the muscle fibers of the gastrocnemius of *Rana pipiens* are typically monosegmentally innervated and that plurisegmental innervation, even as a variation, is rare.

Innervation of the fibers of the sartorius. A study of the distribution of the nerve fibers from different segments gave more variable results in the sartorius. In one series of 21 frogs which were operated on in 1928–29, the anterior root of N X was cut within the vertebral canal in 9 cases and the entire N X outside the vertebral canal in 12 cases. It was found on examining the sartorius of the operated side that there were degenerated nerve endings present in all except one case. The degenerated fibers were usually at the lower pole of the muscle, at the lower pole and along one side, or represented by a single or several bundles of degenerated fibers toward the middle of the muscle. In a few cases they were confined to the upper pole. Usually the degenerated fibers were greatly exceeded in number by normal fibers.

In a second series of 23 frogs studied in 1929–30 the anterior root of N X was cut within the vertebral canal in 4 cases and the entire N X outside the vertebral canal in 19 cases. In not a single instance in this series were any of the fibers innervating the sartorius muscle found to degenerate even though the degeneration time was sufficiently long to cause marked degeneration in nerve-fibers supplying the gastrocnemius.

The lumbo-sacral plexus was examined in 11 frogs of the second series with a view to determining the relative size of the component nerve trunks. In three cases the plexus was found to correspond to the anterior type of

plexus described by Langley and Orbeli (1910) and in 7 cases, to the median type. In the remaining case the plexus on the left side was of the anterior type and on the right side of the median type.

In the anterior type of plexus N VIII about equals in size N X, while N IX is distinctly larger than either. Root XI is very small and does not send a branch to the plexus. In the median type N IX is about equal in size to N X while N VIII is considerably smaller than either of the other nerves. Root XI sends a branch to the plexus. In both types there is considerable variation in the level at which the individual elements join the plexus.

The finding of an anterior or median type of plexus in the frogs in which no fibers from N X supplied *M. sartorius*, suggests, as a possible explanation of the discrepancy between the results in these two series, that a posterior type of plexus may have predominated in the frogs of the first series.

The frogs in which the distribution of N VIII and N IX in the sartorius were studied were from the same group as those in which cutting NX had given no degeneration in the sartorius.

The distribution of N IX in *M. sartorius* was studied in 9 frogs in which that trunk was cut. In every case except two, the great majority of the motor-end plates showed degeneration. In some cases the degenerated fibers were scattered throughout the whole muscle, normal and degenerated fibers running together in some bundles while other bundles were composed entirely of degenerated fibers. In other cases all fibers were degenerated except those supplying the upper pole or one side. In one case the nerve fibers that escaped degeneration were situated at the lower pole.

On cutting root VIII (6 cases) a complementary distribution occurred. Only a small proportion of the fibers present were degenerated. A few degenerated bundles appeared at the upper or lower pole or along one side, or there were scattered degenerated fibers in bundles composed mainly of normal fibers. The degeneration was usually more toward the upper than the lower pole. In only one case did section of N VIII result in no degeneration in M. sartorius.

From the above results it is evident that there is a great deal of variation in the innervation of M. sartorius,—a variation which affects the spinal cord segments from which it derives its supply as well as the distribution within the muscle of the fibers from any particular nerve trunk.

Thirty-four M. sartorii were examined in which there were degenerated and non-degenerated motor-endings present. In 26 of these cases not a single muscle fiber was found on which there ended a degenerated as well as a non-degenerated fiber. In 6 of the 8 remaining cases, one fiber bisegmentally innervated was found. In one case there were two such fibers and in the remaining case three.

All of these 8 cases were in sartorii in which degeneration had resulted from cutting N X.

From the above results it would appear that the fibers of *M. sartorius* as well as the fibers of *M. gastrocnemius* are typically monosegmentally innervated. Plurisegmentally innervated fibers are found, but only rarely.

Discussion. These morphological findings within the sartorius and the gastrocnemius of the frog are in agreement with the results of the majority of the more recent physiological investigations on the question of plurisegmental innervation. While, as an occasional variation, a plurisegmentally innervated fiber may occur,—in no single muscle were there found more than three such fibers,—obviously an insufficient number to serve as a basis for the explanation of the results obtained by Beritoff (1924) and Cattell and Stiles (1924). Studies on heat production and electrical changes indicate the type of innervation described in this study. Some other explanation than the presence of plurisegmentally innervated muscle fibers must be sought for the difference between the sum of tensions developed on separate stimulation of the nerve trunks supplying a muscle and the tension developed on the simultaneous stimulation of the same trunks. Such alternative explanations have been suggested by Fulton (1925), deBoer (1926), Boyd (1926) and Katz (1926).

The results of the study of the distribution in the frog's muscle of the component fibers of the nerve-trunks supplying *M. gastrocnemius* and *M. sartorius* emphasize particularly the great variation which may be expected and which should be taken into consideration in physiological investigations in which these muscles are used. From the results of physiological experiments it has been pointed out by Samijloff and Wassiljewa (1925), deBoer (1926), Cattell (1928) and many others that there is great variation in the spinal segments supplying *M. sartorius* and *M. gastrocnemius*. Boyd (1926) gave up the use of the sartorius as it "proved to be erratic as regards its sources of innervation and unsuited for statistical study." The above results show that variation occurs not only in the segments from which these muscles derive their nerve supply but also in the type of distribution occurring within the muscle. In some cases nerve fibers from different cord segments run in the same bundles and supply muscle fibers in the same region of the muscle while, in other cases, each segment supplies a distinct portion of the muscle.

This variation is much more marked in the frog's sartorius than in its gastrocnemius. In the latter the typical distribution was of fairly constant occurrence, N X supplying a small region in the periphery of the upper pole, N IX supplying the rest of the muscle. This is particularly of interest in view of the finding of Samijloff and Wassiljewa (1925) that stimulation of one root resulted in contraction of the upper part of the frog's gas-

trocnemius and stimulation of the other resulted in contraction of the upper and lower part. deBoer (1926) also found that there were two distinct groups of fibers in the gastrocnemius, a ventral and a dorsal. The ventral, supplied by N IX, was much larger than the dorsal which received its supply from N X.

It is apparent that histological study of frog's *M. gastrocnemius* not only fails to demonstrate the presence of plurisegmental innervation but even shows that the distribution of the nerve endings and the arrangement of the muscle fibers are such that plurisegmental innervation is not possible except in a small constricted region.

The existence of plurisegmental innervation of muscle fibers can only be determined by degeneration experiments of nerve roots with subsequent examination of the loss of the innervation of the muscle fibers themselves. The physiologist who wishes to study the problems of tension, of heat production, etc., in plurisegmentally innervated muscle fibers will have to use muscles other than *Mm. gastrocnemius* and *sartorius* of the frog.

SUMMARY

This histological-experimental study shows that the muscle fibers of the *M. sartorius* and of the *M. gastrocnemius* of the frog are typically monosegmentally innervated. Although plurisegmentally innervated muscle fibers were found in each of these muscles, they are rare. A small region at the periphery of the upper pole of the *M. gastrocnemius* is supplied by N X. The rest of the muscle receives its innervation from N IX. Great variation was noted in the spinal cord segments supplying the *M. sartorius* as well as in the distribution of endings within the muscle. The majority of the nerve fibers innervating the *M. sartorius* are derived from N IX; N VIII or N X supplies the remaining muscle fibers.

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THE RESPONSE OF NEGATIVE AFTER-IMAGES TO PASSIVE MOTION OF THE EYEBALL AND THE BEARING OF THESE OBSERVATIONS ON THE VISUAL PERCEPTION OF MOTION

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Received for publication June 5, 1930

It was reported by one of us (1924) that in photopsia (scotomata) there may sometimes be voluntary fusion of the patches of light with brightness summated, although usually, if there is fusion, it is involuntary without summation of brightness. In attempting to verify this observation under controlled conditions, we found that after-images remained fixed in the visual field when the eyeball was passively moved. This fact is recorded by Helmholtz (1867) who mentions it as proof that direction in the field of vision is not a function of retinal stimulation alone. We shall report in this paper the effect of passive rotation of the eyeball on a variety of entoptic phenomena, as seen by normal eyes and by one aphakic observer, with view to analyzing the optical conditions for the visual perception of motion. The entoptic phenomena investigated were as follows: negative after-images observed during nystagmus and during rotatory and pendular motion of the observer's body; muscae volitantes; foveal figure; retinal circulation; electrical stimulation of the retina; Purkinje's figures; LeCat's experiment and the blue arc. Observation shows that no motion is seen in the first five experiments; but does appear in the last three. In this paper the conditions under which objects appear to move and to remain still are analyzed and the mode of retinal stimulation operating in each case is summarized. The results indicate that the sensation of motion depends upon either successive stimulation of adjacent, retinal, elements or upon kinesthetic impulses of the eye muscles. Since both of these factors are absent in the passive rotation of an eyeball with an after-image impressed upon it, it follows that no motion is seen.

Negative after-images were obtained by fixation for 30 seconds of a 10 watt lamp of a bluish color measuring about 4 by 2 cm. Rotation (8° to 13°) of the eyeball by pressure from the finger at the outer angle of the orbit, produces no motion of the after-image, under any conditions of illumination, although the effect is most striking in a dark room. Slight passive movements of the head do not affect the after-image, whereas larger move-

ments cause it to move. These experiments were also performed in daylight so that the accompanying movements of the eyes might be observed. Passive movements of the whole body pendularly, as in a swing, or circularly, as when the swing ropes are wound up and allowed to untwist, produce corresponding movements of the after-image. During nystagmus induced by rotation (10 times in 1 minute) after a strong after-image of a 60 watt lamp had been obtained, the perception of the after-image is intermittent. The after-image is not seen during the saccadic phase of the nystagmus. Passive displacement of the eyeball during the slow component, that is, during motion in the direction of the rotation which induced the nystagmus, has no effect upon the fixity of the after-image. These observations were repeated on a subject in whom the lens of the right eye had been extracted for traumatic cataract. The cornea and media of the eye are perfectly clear. The retina and nerve head are normal. Vision of the right eye is 20/20 with a plus twelve diopter lens. There is some deviation of the right eye (about 20 degrees) probably because he prefers not to use his lens. He depends entirely upon his left eye which has normal vision. His observation with his normal eye of the effect of passive rotation of the eyeball on the negative after-image confirmed the result already described. In order to obtain an after-image in the right eye, it was necessary to use the twelve diopter lens. Passive rotation of the right eyeball caused no motion of the after-image. The phenomenon is, therefore, independent of the lens.

Passive motion of the eyeball does not cause movement of muscae volitantes. The corpuscles of the retinal vessels may be made apparent, as swiftly darting particles, by fixating a bright surface through cobalt glass. These particles do not move with passive motion of the eyeball. The foveal figure appears as an orange tinted, roughly circular, spot in the center of the greyish field of granules and branching figures when a bright white surface, is fixated through a circularly moving pinhole. Passive motion does not displace it nor the other elements surrounding it.

Visual sensations due to electrical stimulation of the retina were produced by a current from an inductorium, sent through the head. The large, indifferent, electrode was placed on the left side of the head and held in place by a fiber band. The small electrode was applied to the region of the right orbit. The electrodes were saturated with sodium chloride solution. With proper conditions of contact and current in a dark room, a yellowish green light is seen in the periphery of the field of vision. On rotating the eye with the tip of the finger, no motion of the light is seen. In the five observations mentioned, no displacement of the visual phenomenon occurs with passive motion of the eyeball. The condition of excitation which is common to these five experiments is a stimulus which does not travel from one retinal locality to another.

Three experiments now follow in each of which there is movement of the entoptic appearance with passive motion of the eyeball. In two of these

experiments, the principle of excitation is the same, namely, a shadow is cast upon the retina by some object external to it. In one case, LeCat's experiment, (1740) the object is the head of a pin which is held so close to the cornea that no image is formed by the lens. However, the shadow cast by the pin acts as a stimulus and an inverted image of the pin head is projected into the field of vision. This experiment was also performed by the aphakic observer who used the twelve diopter lens. Without instruction as to what he should see, he drew a picture showing the inverted pin head in the circle of the pin hole. The pin head and the hole were sketched considerably larger with the aphakic than with the normal eye. In the second of these experiments (Purkinje's figures) the shadow is cast on the retina by blood vessels in the choroid by focussing a strong light on the sclera. The magnified, branching, vessels are seen projected in the field of vision.

Observations were made of the blue arc by both the normal and the aphakic individuals. In a dark room the observer fixated with his right eye at a distance of one meter, a brightly illuminated red strip 5×1 cm. The fixation point with the right eye was one centimeter to the right of the mid point of the strip. Under these conditions the flattened elliptical blue arc is seen extending to the right of the fixation point. One may see the blue arc of both eyes simultaneously, by looking at the mid-point of the red strip with both eyes open. In this case the two arcs resemble in shape a pair of flattened spectacle rims open at the outer ends. The form of the arc may be changed by pressure on the eyeball. In one case, the lower member of the arc appeared irregular in outline and in another it had a sharp point or cusp in it. The size of the arc can be altered and it can be made to assume a flatter or wider form, by appropriate pressure. The aphakic observer with the right eye corrected by the plus twelve diopter lens, obtained a clear image of the strip and saw the blue arc which he sketched with a pencil in such a way as to leave no doubt that he observed the same phenomenon as ourselves. With passive rotation of the eyeball, the red strip moved dragging the blue arc with it.

The condition of excitation which is common to these three experiments is a stimulus which travels from one retinal locality to another. We may, therefore, conclude that the sensation of motion is aroused by successive stimulation of adjacent elements of the retina. This may be referred to as the spatial gradient of stimulation of the retina. Relative speed is conditioned by the abruptness of the temporal gradient of stimulation.

The anatomical basis of these gradients may be conceived of in terms of nerve elements, in the case of the retina, on the evidence furnished by Adrian's work (1927) on the action current of the optic nerve. In the case of the kinesthetic impulses, the fractional response of muscles extending even to the individual fiber, affords a physiological ground for a gradient in

this sphere. The work of Sherrington, Sybil Cooper and Denny-Brown (1926) and also that of Pratt (1925) may be cited in this connection.

Consideration of the conditions under which an object appears to the eye to move or to remain still, shows clearly that there is another factor involved, viz., the kinesthetic impulses aroused by contracting muscles. An examination of all types of visual motion, illusory or otherwise, shows six different situations in which objects are seen to move whether they really do or not and three situations in which they remain still. Some of these conditions have been discussed by James (1890). A more complete classification which appears to include all instances of seen motion and seen immobility is as follows.

Conditions under which objects appear to move:

1. Object moves with the eye still. Spatial gradient in the retina.

2. Moving retinal impression with passive rotation of the eyeball. (Purkinje's figures.) Spatial gradient in the retina.

3. Object still with eye really moving but not felt to move. (Nystagmus.) Spatial gradient in the retina.

4. Object still with eye really still but felt to move. (Paralysis of rectus externus.) Kinesthetic impulse.

5. Object moves with the eye pursuing. Kinesthetic impulse.

6. Fixed retinal impression with active motion of the eyes. (After-image moving in space with voluntary movement of the eyes). Kinesthetic impulse.

Conditions under which objects appear to be stationary:

1. Object is still, with active motion of the eyes. The ordinary field of vision. The suggested motion of the object due the spatial gradient of the retina, is inhibited by the kinesthetic impulses from the contracting eye muscles.

2. Object is still with felt fixity of the eyes. No spatial gradient in the retina and a steady kinesthetic impulse.

3. Fixed retinal impression with passive motion of the eye. No spatial gradient in the retina and no kinesthetic impulse.

The study of visual motion shows that two factors interact, viz., the point to point stimulation of the retina which furnishes the spatial gradient and the kinesthetic impulses which are graded on the basis of fractional contraction. The relative importance of these two factors is at present undetermined.

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THE EFFECT OF EPINEPHRIN ON MUSCLE GLYCOGEN IN THE ABSENCE OF THE LIVER, AND A MODIFICATION OF THE OPERATION FOR LIVER REMOVAL

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Received for publication June 7, 1930

In a previous paper (Eadie, 1930) experiments were described which demonstrated that after epinephrin there was a decrease of muscle glycogen in the rat and very slight or no changes in the cat. At the same time the liver glycogen of the cat was decreased while that of the rat was increased. The problem arose whether the absence of a decrease in the muscle glycogen of the cat was connected with the marked fall of liver glycogen in this animal. If this were the case one would expect that if the liver was not acting there would be a decrease in muscle glycogen. There would thus be a peripheral action of epinephrin on carbohydrates, marked in the case of the cat by a predominating central action. It is the purpose of these experiments to investigate this question.

METHOD. The anesthetic used was ether for the most part, but for some experiments Amytal was given subcutaneously. Blood sugars were estimated by the method of Hagedorn and Jensen, and glycogens by Pflüger's method. Samples of blood and muscle were taken immediately after removal of the liver, then epinephrin was given to the treated animals. After about an hour further samples of blood and muscle were taken, in this case using the corresponding muscle from the opposite side. Glycogen is reckoned as glucose.

Operative technic. In 1929 Firor and Stinson described a method for the total extirpation of the dog's liver in one stage. The essential feature of the procedure was the insertion of a pyrex cannula to conduct the portal and vena caval blood to the thoracic portion of the inferior vena cava. Further experience has led to modifications of the technic. Since, however, these improvements are embodied in the method as applied to cats, we will defer a separate description of them.

The technic for the total extirpation of a cat's liver in a one-stage operation has not been previously described. It is as follows: Fullgrown healthy animals are the ones of choice. Under ether anesthesia or with amytal injected subcutaneously a midline abdominal incision is made.

The peritoneal fold between the liver and the right kidney is divided and a loop of braided silk is passed around the vena cava directly above the right adrenal. The portal vein is then dissected out by dividing the hepatic artery, lymphatics, and bile ducts which accompany it. In many cases there is a small accessory portal vein running from the duodenum to the liver. When this is present it is also divided and ligated.

The liver is retracted from the diaphragm and the small vessels crossing from the diaphragm to the liver are cut and tied. A loop of braided silk is passed completely around the vena cava extending between the liver and the diaphragm. In the cat this procedure is extremely simple because the liver can be retracted sufficiently to permit the step to be done under direct vision, and it is furthermore facilitated by the existence of an

TABLE 1

EPINEPHRIN							CONTROLS						
Experiment no.	Anesthetic	Time	Blood glucose		Muscle glycogen		Experiment no.	Anesthetic	Time	Blood glucose		Muscle glycogen	
			Before	After	Before	After				Before	After	Before	After
			mgm. per 100 cc.	*mgm. per 100 cc.*	*per cent*	*per cent*				*mgm. per 100 cc.*	*mgm. per 100 cc.*	*per cent*	*per cent*
37	Amytal	47	84	54	0.63	0.77	36	Amytal	55	30	20	1.04	0.86
39	Ether	55	194	91	0.92	0.79	38	Ether	60	185	72	0.84	0.69
41	Ether	60	144	77	0.67	0.45	40	Ether	71	229	136	0.90	0.60
43	Ether	75	207	140	1.12	0.94	42	Ether	85	177	61	0.60	0.66
45	Ether	58	189	156	1.11	0.98	46	Ether	84	224	84	0.54	0.46
48	Ether	65	154	81	0.54	0.29	47	Ether	66	163	125	0.45	0.32
50	Amytal	60	132	54	0.59	0.48	49	Amytal	63	91	54	0.72	0.54

avascular space covered only by peritoneum, extending between the posterior aspect of the liver and the crura of the diaphragm.

The next step in the operation calls for the cutting off of the portal circulation by placing a Carrel clamp on the vessel at a distance of 1 to 2 cm. from the liver. This vein is then clamped and divided at its point of entrance into the liver. The short arm of a pyrex cannula is inserted into the stump of the portal vein and securely fastened by a ligature of braided silk. Following this step the vena cava is clamped off just above the lumbo-adrenal veins, and it is also occluded just below the diaphragm by making traction on the encircling silk ligature. The vena cava is scraped free of overlying liver tissue and is incised longitudinally for a distance of 2 cm. The cannula is filled with normal salt solution and inserted into the vena cava. When the upper end of the cannula encounters the taut silk ligature occluding the diaphragmatic portion of the

vena cava, this ligature is momentarily relaxed and the cannula pushed beyond. The encircling loop is then drawn securely around the vena cava and the cannula.

The lower end of the cannula has up until this time contained a small cork, which prevents leakage. This cork is removed, and with his index finger over the opening the operator grasps the cannula by the portal arm and slips the open end of the cannula into that portion of the vena cava which extends below the longitudinal slit. Here again there is an encircling ligature of braided silk which is easily adjusted and securely tied around the tube and the vessel. The Carrel clamp on the portal vein and the clamp occluding the vena cava are immediately removed, and the circulation is restored. As a matter of fact, the Carrel clamp is sometimes taken off as soon as the upper end of the cannula is in place. The complete excision of the liver is accomplished by dissecting the liver and the vena cava from around the cannula. The wound is not closed until complete hemostasis has been assured.

This operation has been done in 20 minutes; it usually takes about 30 minutes. The animals regain consciousness shortly after the operation is ended. Unless glucose is given they die in from one to three hours with hypoglycemic symptoms. In one case we made an effort to keep the cat alive by injecting a 2 per cent glucose solution through a jejunostomy tube every hour. This animal lived nine hours.

The cannulae vary in length from 4.5 to 5.5 cm. Their bores are from 3 to 4 mm. The portal arm is from 2 to 4 mm. in diameter and is placed from 0.5 to 1.0 cm. from the lower end of the cannula. The maries on the cannula have been replaced by small flanges, which prevent the ligatures from slipping off. By reference to the article by Firor and Stinson in the Johns Hopkins Hospital Bulletin it will be seen that the technic has been modified by attaching the cannula to the portal vein before inserting it into the vena cava instead of after. A second important improvement has been in choosing a cannula which is about one half the size of the vessel rather than using one which nearly equals the diameter of the vessel.

We have attempted the operation 20 times in cats and have failed on 4 occasions; once because of an anomaly in the portal vein, twice because we tore this same vein after the cannula was in place, and once because clotting occurred from pinching off the portal vein when inserting the lower end of the cannula. The operation in cats is much easier than in dogs, chiefly because one gets better exposure and because the liver can be easily retracted from the diaphragm.

Results. The results are given in the accompanying table. "Time" refers to the interval between removal of the muscle samples. The time for the blood samples differs from this only by a minute or two. The points to be noticed are that both controls and treated animals show

approximately the same decreases of blood sugar. In both sets the muscle glycogen decreases and to about the same extent in each, with two exceptions only one of which is a control.

DISCUSSION. These experiments show no sign of peripheral action of epinephrin, either on muscle glycogen or on the rate at which glucose leaves the blood. The latter is a confirmation of the work of Soskin (1927).

These results differ from those of Cori and Cori (1929) on the rat and this may be so for two reasons. In the first place the technic used by these authors and presumably adopted because of the size of the animals is not, as they point out, capable of giving conclusive results. In the second place, as pointed out by one of us (Eadie, 1930), there exists a marked difference between the rat and the cat.

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SOME CONDITIONS AFFECTING THE RESPONSES OF LIMULUS HEART TO ARTIFICIAL AND NATURAL STIMULATION

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Received for publication May 24, 1930

That there is a greater difference between the effectiveness of artificial and natural stimulation of tissues than is generally recognized has been demonstrated by one of us (Asher), who found the frog's heart to be a suitable object for this purpose.

Owing to the segregation of nervous and muscular tissue in the heart of Limulus it is possible to analyze the nervous and muscular elements in its reactions, and for this reason it seemed desirable to extend the studies on natural and artificial stimulation to this experimental object which has been of such distinct value for the analysis to the physiology of the neurogenic heart. The experiments were conducted at Woods Hole during the summer of 1929.

The experimental approach was similar to that in Asher's experiments with the frog's heart. Briefly stated: when the frog's heart was subjected to a complete lack of oxygen the responses to artificial stimulation of the ventricle progressively diminished to extinction although the strength of the stimuli delivered by a standardized Kronecker induction coil was definitely increased in the course of the experiment. Notwithstanding the failure to respond to these artificial stimuli, the normal stimuli from the sinus caused the ventricle to contract and to perform a measurable amount of work. In a similar manner the superiority of natural stimuli, and possibly even their qualitative difference from artificial stimuli could be demonstrated by carefully graded poisoning of the frog's ventricle with a solution of potassium chloride. At a certain phase in the action of this solution there was no response to artificial stimuli of even high intensity but the natural stimuli continued to be effective.

Effects of potassium chloride. Similar studies on the effects of potassium chloride were made on the Limulus heart prepared for experimentation in the general manner described by Carlson (1). The ganglionic pace-maker was immersed in one vessel containing sea water, while the muscular seg-

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ments were submersed in another container and their contractions recorded in the usual manner by light levers writing on a smoked drum. Two solutions of potassium chloride were prepared, one containing 2.4 grams per liter, the other 4.8 grams per liter, both made up in sea water. Sea water alone is a satisfactory balanced saline in which the heart continues to beat for many hours. It was found that when the muscular segments were treated with the sea water containing the lesser amount of potassium chloride, i.e., an excess of this salt amounting to 2.4 grams per liter, the automatic contractions showed no deleterious effects. Since the pace making ganglion remained in pure sea water it is evident that the muscular tissue of the Limulus heart is resistant to an increase in the amount of potassium chloride considerably above that normally present in sea water and that the automatic normal impulses continue to be effective. But when the muscular segments were immersed in sea water to which the double quantity of potassium chloride had been added (4.8 grams per liter) the contractions ceased (fig. 1) as Carlson has previously reported. The normal automatic stimuli failed to be effective. When, however, these quiescent muscular segments were artificially stimulated by induction shocks, especially by tetanizing shocks applied through platinum electrodes connected with the secondary coil of a Harvard inductorium, it was found that they were as excitable to these stimuli as before treatment with the potassium chloride. While we may conclude then that there is a difference in the responses to artificial and normal stimuli it must be noted that the result is the exact reverse of that shown by the frog's heart similarly treated with potassium chloride, i.e., for the Limulus heart artificial stimuli are the more potent, for the frog's heart natural stimuli, as shown in the Bernese laboratory by Bachmann (2). These two, equally exact but opposite, results should serve as a deterrent against too rash a generalization from one field of physiological phenomena to another, in which the reactions may be of an entirely different order. Two tracings showing the results of the above experiments on Limulus heart are shown in figure 1. Potassium chloride solutions were applied to the anterior segments of the beating Limulus heart at x and x'. After the contractions had disappeared tetanizing currents were applied at y and y' respectively and it is to be noted that these artificial stimuli caused effective contractions although the normal ganglionic impulses were impotent.

That strong solutions of potassium chloride suppress the automatic contractions while more dilute solutions have no harmful effect has been stated above. It was found that under certain conditions a concentration of 2.4 grams per liter of sea water instead of being harmful actually may be beneficial to the autogenous action of the Limulus heart.

This is demonstrated by experiments of the type illustrated in figure 2 (A and B) in which the automatic contractions had been gradually weak-

ened by the prolonged action of pure carbon dioxide which, after washing, was bubbled through the sea water in which the contracting segment was immersed, then at the points K the potassium chloride was introduced (2.4 gm. per liter) and immediately there ensued a marked augmentation of the contractions which was definitely in excess of the contractions in pure sea water either before or after the experimental procedures and potassium chloride may be regarded as an antagonist to the harmful effects of carbon dioxide. The augmentor effects are so certain and so pronounced that this

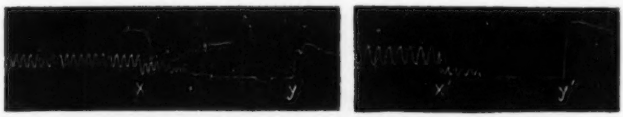

Fig. 1. Two tracings of Limulus heart, showing that potassium chloride (4.8 grams per liter) applied at x and x' suppresses automatic contractions. Faradic currents applied at y and y' were still effective.

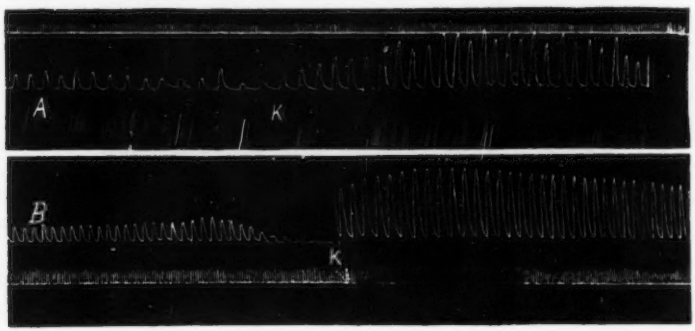

Fig. 2. Weak contractions due to the action of CO_2 are shown at the left of both tracings A and B. The application to the muscle of KCl (2.4 grams per liter) at K caused marked augmentation of the muscular responses to normal neurogenic impulses.

potassium solution was frequently used in experiments in which contractions were so weakened by other conditions that it was desirable to restore them to some semblance of normality. One is reminded upon observing these effects of potassium that they are in some measure similar to the effects repeatedly reported by Zwaardemaker, namely, that under proper conditions potassium is an activator of the heart beat; this activation he attributes to α and β rays emanated by potassium. The school of Rothberger maintains that part of the action which Haberlandt attributes to his heart hormone is due to the action of potassium, a concept which may

also well be brought into consideration in the cardiac augmentation which, according to Enid Tribe Oppenheimer (3) is obtained by extracts of a number of widely differing tissues.

Effects of carbon dioxide. One of the means used to weaken the heart beat and to determine whether, in the resulting weakened state, natural or artificial stimuli are the more potent, was to bubble pure washed carbon dioxide through the immersion fluid. The method used was modified from the description by Carlson (loc. cit.) and by Newman (4). The arrangement of two troughs permitted independent treatment of either the ganglion or the muscle with the gas. The action of the gas on the ganglion alone was attended with little change in the contraction of untreated

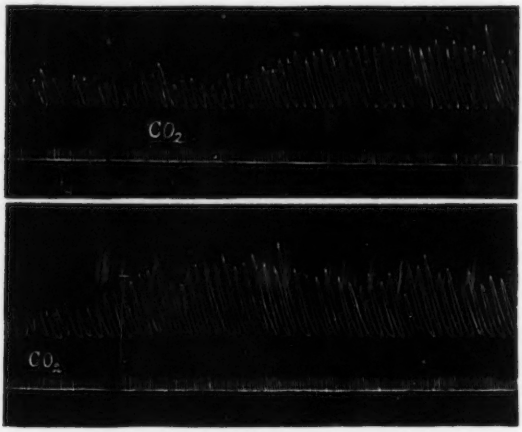

Fig. 3. Two tracings showing augmentation of the muscular contractions of Limulus heart due to carbon dioxide gas applied to the muscle at CO_2.

muscle, often for a considerable time, then quite suddenly the contractions became seriously impaired. Newman (loc. cit.) records immediate impairment. The muscular response to carbon dioxide is quite the reverse of that of the ganglion. When the muscle alone was subjected to the action of carbon dioxide it responded for a time with a decided increase in the strength of its contractile response to normal stimuli and this muscular augmentation was likewise immediately in evidence when the whole heart was treated with carbon dioxide. In the latter case the muscular augmentation is prominent in spite of the demonstrated impairment which the ganglion may suffer. Of the numerous experiments illustrating the effects just described, figure 3 presents two tracings showing the immediate augmentation resulting when perfusion with carbon dioxide was begun at CO_2.

The tracings resemble in appearance the effects of stimulation of the augmentor nerves of the frog's heart and this comparison suggests that the explanation of the action of the augmentor nerves, as well as that of Loewi's alleged accelerans hormone may actually be due, at least in some measure, to a readjustment in the concentration of the hydrogen ions as Atzler and Müller (5) have already suggested, indeed it has long been established that slight changes in carbon dioxide content and in the hydrogen ion concentration of the blood have definite effects on the respiratory and cardio-vascular system. Neither these effects nor those of potassium considered above can be overlooked in this connection. In the above experiments we are dealing with an increase in the hydrogen ion concentration of the sea water in which the heart is immersed, but the initial concentration of which is well on the alkaline side of neutrality (pH 8 or 8.2). The augmentor effects, as already stated, are due to the action of carbon dioxide on the muscle alone and not to action on the impulse forming gan-

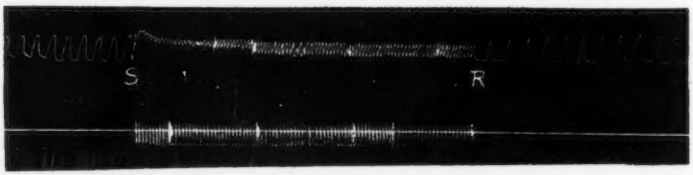

Fig. 4. Normal autogenous contractions are interrupted at *S* by induction shocks delivered one per second. The slight "apparent fatigue" disappears with resumption of the normal slow rate at *R*.

glion cells which are never affected in this manner. This means that at a certain concentration, carbon dioxide, or the resulting pH, so changes the conditions of the muscle that it responds more effectively to the normal fixed strength of stimulation sent out by the ganglion—an interpretation which is the only one possible if we assume the validity of the "all or none" law. In principle then carbon dioxide acts in the same way as an increase in the initial length of the muscle fiber or as a change in metabolic state, both of which conditions may cause a stronger reaction to stimuli of constant strength.

The augmentor effect of carbon dioxide passes off after some time if the perfusion is continued and sooner or later the depressing effect of this agent appears, as has been noted in the description of the preceding experiments. For the purpose of control washed hydrogen was substituted for carbon dioxide in the above experiments, but no augmentor effect was ever observed by us or by Newman (loc. cit.) in his earlier experiments.

Fatigue. In several papers from the Bernese laboratory it has been shown that when skeletal muscle of the frog or of a mammal, or the heart

muscle of the frog, is brought to the state of fatigue by stimuli repeated in rapid sequence, this fatigue can be abolished immediately by shifting, without any pause, to a slower rate of stimulation. This type of fatigue therefore was designated "apparent fatigue." It was of interest to continue the investigation of this phenomenon on the Limulus heart. Platinum electrodes were applied to the ganglion. Induction shocks from a Harvard inductorium were used as stimuli and rates of stimulation of 30 and 60 per minute were obtained by interrupting a dry cell in the primary circuit with a Harvard-Bowditch pendulum clock. When the automatic beat was interrupted by artificial stimulation at the rate of 60 per minute the heart contracted in response to each stimulus (fig. 4, *S*); when the artificial stimulation ceased the normal automatic rhythm was immediately resumed. An hour of this artificial stimulation at the rate of 60 per minute caused symp-

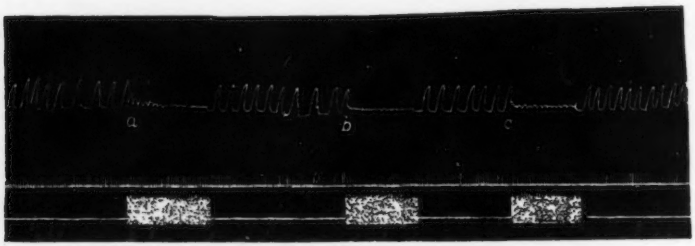

Fig. 5. Contractions of the Limulus heart at the end of one hour of artificial stimulation of the muscle. The normal neurogenic contractions are interrupted at *a*, *b* and *c* by induction shocks at the rate of one per second. The evidence of "apparent fatigue" is absent when the normal slow rate of contraction is resumed.

toms of fatigue, but when the rate of artificial stimulation was cut down to 30 per minute the heart followed at this rate and the contractions immediately became higher. With cessation of artificial stimulation and resumption of the automatic rhythm all the evident characteristics of fatigue disappeared from the tracing. Figure 5 is a record of the cardiac contractions after one hour of artificial stimulation of the ganglion with 60 stimuli per minute. The automatic contractions appear normal but three stretches of artificial stimulation, at *a*, *b* and *c*, show the incidence of fatigue with each stimulation at the rate of one stimulus per second and its disappearance as soon as the automatic slower rate is resumed. These, and other types of experiments prove that in the Limulus heart, as in the frog's heart, the state of fatigue is only "apparent fatigue" and demonstrate the high degree to which this heart is able to resist fatigue when subjected to such rates of stimulation as the ganglion is capable of developing. Especially is this noteworthy when one considers how abnormal the meta-

bolic state of such an excised heart must be as compared with that of a well perfused frog's heart.

CONCLUSIONS

1. If the Limulus heart be weakened, for instance by poisoning with potassium chloride, artificial stimulation is still very effective, while the natural impulses are not. This is the reverse of the results obtained on the frog's heart, where natural excitations are still highly effective when even the strongest of artificial excitations have no effect at all.

2. A solution containing 2.4 grams potassium chloride per liter of sea water has a strong augmentor effect on a weakened heart beat. Even hearts that have practically ceased to beat may be restored by this solution, an influence of a potassium salt which is similar to that reported by Zwaardemaker.

3. In a certain phase of its action on the Limulus heart CO_2 exerts a powerful augmentory effect on the contractions; the point of attack of this augmentation is on the heart muscle and not on the ganglion. It resembles the action of the augmentor nerves of the heart and it supports the criticism against the existence of a specific substance underlying the effects of excitation of the augmentor heart nerves.

4. When in the Limulus heart a state of fatigue is produced by an artificial, rapid rate of stimulation, this fatigue disappears immediately with abolition of the rapid rate of stimulation and the heart follows the slower normal excitations without any evidence of fatigue. Therefore there exists also in Limulus heart the state which may be designated "apparent" fatigue.

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PHOSPHOCREATINE AS A BUFFER IN MAMMALIAN MUSCLE

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Received for publication June 7, 1930

In 1928 Fiske and Subbarow (1) suggested on theoretical grounds that phosphocreatine could act as a buffer in muscle, the hydrolysis of 0.45 per cent of phosphocreatine (the average amount in resting cat muscle) liberating sufficient base under optimum conditions (pH 6) to neutralize the lactic acid formed up to a concentration of about 0.23 per cent. They calculated that "approximately half this amount of lactic acid could be neutralized at pH 7, i.e., without the development of any acidity at all."

In another paper the present authors (2) reported the changes in lactic acid and labile phosphorus in resting and exercised frog muscle, and they were unable to demonstrate any definite quantitative relationship between the disappearance of labile phosphorus and the production of lactic acid.

In the present investigation we have used cats, and have made direct measurement of the hydrogen ion concentration simultaneously with the chemical changes taking place in muscle *brei* made from resting muscle.

The experiments were done on decapitate cats, using the technique of Simpson and Macleod (3), in which the gastrocnemii were first separated from adjacent tissues, but origins, insertions, blood and nerve connections left intact, the animal then allowed to rest for approximately 4 hours and then the muscles quickly excised and frozen in liquid air. Owing to the fact that the fleshy parts of the gastrocnemii were cut across in excision our initial lactic acid values are higher than those obtained when the semimembranosus and semitendinosus were used and only the tendinous portions actually cut (3). The frozen muscle was ground to a fine powder, weighed out in previously cooled weighing bottles, and returned to a mixture of ice and salt. To each muscle sample of about one gram, 5 cc. of distilled water were added and the bottles then placed in a water bath at constant temperature. Samples for analysis were taken at various intervals from three minutes to two hours. Lactic acid was estimated according to the method of Friedman, Cotonio, and Shaeffer (4), phosphocreatine by the method of Fiske and Subbarow. The samples for pH determination were rapidly centrifuged and the hydrogen ion content of the supernatant

fluid found by use of a quinhydrone electrode.[1] From time to time the quinhydrone electrode was checked against solutions of known pH.

The average amount of phosphocreatine found in resting cat's muscle in our experiments was lower than that found by Fiske and Subbarow,

TABLE 1

10°C.

P.C. = phosphocreatine; L.A. = lactic acid

CAT NUMBER	NORMALS			3 MINUTES			6 MINUTES			9 MINUTES			12 MINUTES			1 HOUR			2 HOURS		
	P.C.	pH	L.A.	P.C.	pH	L.A.	P.C.	pH	L.A.	P.C.	pH	L.A.	P.C.	pH	L.A.	P.C.	pH	L.A.	P.C.	pH	L.A.
1	50.6	7.28	0.15	26.6	7.30	0.23	21.0	7.53	0.26	10.6	7.53	0.25	13.8	7.45	0.27				2.8	6.5	
2*	42.6	7.45	0.18	17.2	7.57	0.23	15.0	7.45	0.35	9.6	7.47	0.35	8.4	7.35	0.40						
	35.0	7.55	0.14	9.8	7.55	0.16	6.4	7.50	0.26		7.45	0.42	9.4	7.28	0.37						
3	41.8	7.28	0.10	23.8	7.53	0.135	8.4	7.53	0.16	5.0	7.53	0.20	0.0	7.37	0.23	0.6	7.03				
	40.4	7.60	0.09	4.2	7.58	0.12	5.0	7.57	0.13	8.0	7.45	0.19	7.2	7.48	0.22		7.20				
8		7.37		19.8	7.45		13.5	7.43		10.2	7.39									7.03	
	43.2	7.45		17.8	7.49		8.0	7.49		5.7	7.28									6.86	
Aver.	45.6	7.42	0.13	17.0	7.49	0.175	11.5	7.50	0.23	8.2	7.44	0.280	7.7	7.38	0.300	0.6	7.12		2.8	6.80	

* First figure for first leg; second figure for second leg.

TABLE 2

25°C.

P.C. = phosphocreatine; L.A. = lactic acid milligram per gram

CAT NUMBER	NORMALS			3 MINUTES			6 MINUTES			9 MINUTES			12 MINUTES			1 HOUR			2 HOURS		
	P.C.	pH	L.A.	P.C.	pH	L.A.	P.C.	pH	L.A.	P.C.	pH	L.A.	P.C.	pH	L.A.	P.C.	pH	L.A.	P.C.	pH	L.A.
4	39.9	7.37	0.10	0.0	7.28	0.19	0.0	7.28	0.23	0.0	7.28	0.28		7.28	0.32		7.11	0.35			
5	46.0	7.53	0.06	10.5	7.45	0.275	16.4	7.45	0.36								7.03	0.59		6.94	0.59
	50.8		0.10	9.2	7.61	0.29	6.3	7.45	0.38								7.25	0.60		6.89	0.62
6	41.4	7.53	0.09	5.7	7.32	0.32	4.7	7.28		5.8	7.20	0.39					6.95	0.45		6.60	
	38.7	7.61	0.11	2.5	7.20	0.32	2.0	6.95	0.32	4.6	6.95						6.78	0.45		6.55	0.38
7	56.0	7.45	0.11	6.2	7.28	0.22	8.4	7.15	0.25	3.8	7.08	0.30					7.03	0.35		6.81	0.36
	39.9	7.50	0.08	9.0	7.28	0.16	7.0	7.22	0.19	7.7	7.06	0.23					6.94	0.30		6.81	0.32
Aver.	45.0	7.41	0.098	6.2	7.35	0.255	6.4	7.25	0.290	4.4	7.11	0.300		7.28	0.32		7.01	0.44		6.77	0.45

i.e., 0.45 mgm. inorganic phosphorus as against 0.60—0.70. This is without doubt due chiefly to two factors: 1, the use of decapitate cats (cf. Davenport and Davenport and Ranson (5); 2, the preliminary manipula-

[1] We wish to acknowledge our thanks to the Board of Research of the University of California for funds to purchase the quinhydrone electrode.

tion in freeing the gastrocnemius, and the final cutting across the fleshy part of the muscle in removal. The result of both these procedures is to cause the initial lactic acid to be slightly over 0.1 per cent (average of all experiments, 0.11 per cent) instead of the theoretical 0.06 per cent.

Two groups of experiments were performed, one at 25°C. and the other at 10°C. The individual results are shown in the tables and the averages graphically in the figures. The data obtained when the *brei* was kept at 25° shows that within the first 3 minutes there is a sharp rise in lactic acid to about 0.25 per cent, the phosphocreatine almost entirely disappears,

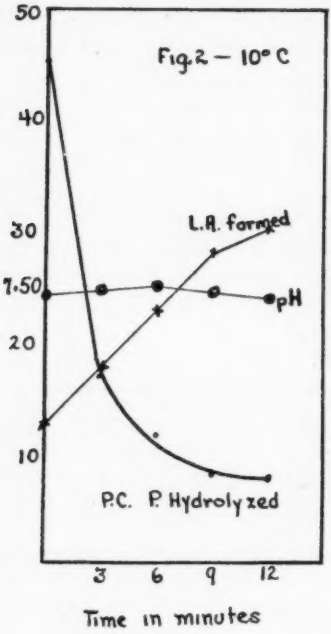

Fig. 2

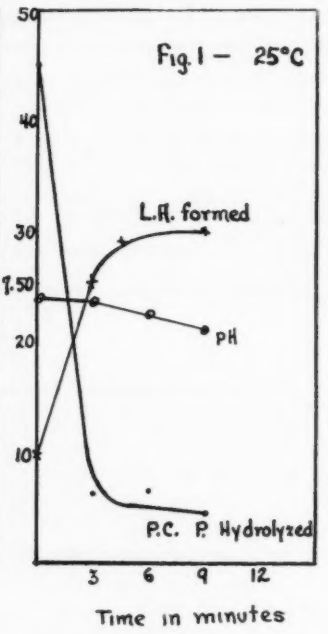

Fig. 1

but the pH declines only slightly. In figure 1 the one aberrant pH, viz., 7.95, in cat 5 was omitted in calculating the average. In practically none of the 3, 6, and 9 minute samples was there a change in the phosphorus readings within the first 5 minutes after addition of the reagents, indicating that no labile phosphorus was present, but upon standing the half-hour for the final reading there was almost invariably a color change, so that in spite of the lack of color change within the critical 5 minute period the initial and final values were not exactly the same. This slight amount of

inorganic phosphorus which appears during the half-hour may very well come from some source other than phosphocreatine. It will also be seen that the pH falls somewhat more sharply after the first 3 minutes following nearly a straight line, while the rate of lactic acid production appears to slow up somewhat after the first 3 minutes.

When the muscle *brei* is kept at 10° instead of 25° the result appears to be strikingly different. The phosphocreatine falls off gradually so that at the end of 6 minutes there is still approximately 25 per cent left, the lactates meanwhile rise in an almost straight line to a value of about 0.23 per cent, and the pH does not decrease. Up to this point the muscle suspension evidently has an adequate buffer mechanism. But beyond this point the pH begins to fall, the lactic acid continues to increase at a fairly uniform rate, and the phosphocreatine decreases slowly.

CONCLUSION

Comparison of the data shows 1, there is a buffering effect in mammalian (cat) muscle *brei* until approximately three-quarters of the phosphocreatine has disappeared; 2, the breaking point in the buffering action occurs when the lactic acid has reached a concentration of approximately 0.25 per cent.

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THE CARBOHYDRATE METABOLISM OF THE HEART

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Received for publication May 20, 1929

A balance experiment on the relation between the carbohydrate disappearance and total metabolism of the isolated heart should give information as to whether carbohydrate is the sole source of energy for the recovery process in muscle contraction. Locke and Rosenheim (1907) did this in two experiments, measuring the carbon dioxide elimination of the isolated heart, and found glucose disappearing in an amount nearly equivalent to the amount of carbon dioxide eliminated. Their experiments were conducted with a large excess of carbohydrate in the system and with saline perfusion. Perfusion with saline saturated with oxygen at atmospheric pressure can convey to tissues only a small fraction of the amount of oxygen which a blood system can convey, and hearts so perfused are in reality working under severely anaerobic conditions. Gayda (1911) also made such measurements and observed a glucose disappearance several times greater than the carbon dioxide production. In these experiments too the poor oxygenation of the preparation prevented the normal oxidative recovery, and an accumulation of lactic acid probably accounts for the very large glucose loss. A more carefully controlled study of this relationship seemed desirable and has therefore been carried out.

The study of carbohydrate utilization by a beating heart is complicated by the fact that not only is the heart removing sugar from the fluid perfusing it, but under some circumstances (as under anaerobic conditions) the stored carbohydrate of its own tissues disappears. If the oxygen consumed is quantitatively great enough to make small changes in glycogen insignificant the heart can be used for such studies. The heart-lung preparation is particularly useful because by it the recovery process may be studied over a long period of time at normal oxygen tension, allowing the muscle to carry on the cycle of breakdown and restitution processes a very large number of times. The proportion of the total metabolism due to the lungs has been investigated by Evans and Starling (1913) who found that approximately 20 per cent of the total metabolism, while the heart was doing moderate work, was referable to the lungs and the blood itself. When greater loads were put upon the heart muscle the proportion of the total metabolism due to the lungs decreased, inasmuch as the work of the heart had

no effect upon the lung metabolism. Whether there are qualitative differences in the metabolism of the two tissues is unknown.

METHOD. Heart-lung preparations were made, following essentially the methods of Knowlton and Starling (1912) as modified in a few details by Starling and Visscher (1927), and further modified as regards the system of ventilation as described below. The blood temperature was maintained at 37°C.

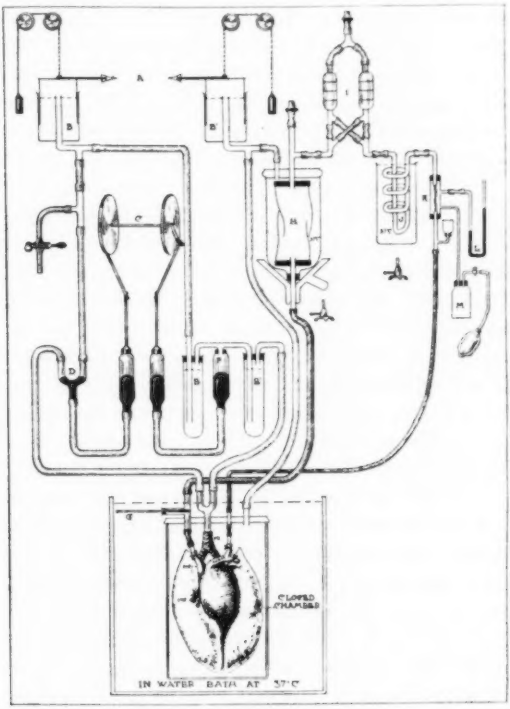

Fig. 1. Scheme of working system. Description in text

with variations of no more than 0.5°C. The chamber enclosing the heart-lung was about three-fourths submerged in a water thermostat, set at 37.0°C. A Stolnikov stromuhr was inserted in the external circuit (*I*, fig. 1) in order to fix the output of the heart at a figure around 250 cc. per minute. The total output will differ from this figure by the amount of the coronary circulation. In these experiments we had no concern regarding the work done by the heart other than in keeping it at a reasonable level, and have consequently not measured the coronary circulation.

The oxygen consumption of the heart-lung preparation was measured by the arrangement described by Visscher and Mulder (1930). The system (fig. 1) is a recording spirometer, *B*, in connection with a closed system mercury respiration pump ventilating the lungs. The pump draws oxygen from a small spirometer of about 200 cc. capacity through a water valve, *E*. The lungs are filled by the pump through the water valve, *E'*, and they empty themselves at the end of inspiration through a trap mercury valve, *D*, which opens at that time, the expired air being blown through a soda lime tower in line from *D* to *B*, but not shown in the diagram, into the spirometer, *B*. The spirometer carries a writing lever which records its level on a large kymograph at *A*. The rate of fall of the spirometer is determined by the oxygen consumption of the heart-lung preparation, so long as the elastic properties of the lung remain unaltered.

The elastic tension of the lung wall alters during an experiment; we have therefore used the system for measuring the increase in residual air capacity of the lungs shown in figure 1. Since edema of the tissues and stasis of blood in certain vessels complicate the true dilatation it was necessary to take account simultaneously of the loss of blood from the collapsible reservoir, *H*. This is accomplished by connecting by air channel the air chamber around the collapsible reservoir with the closed chamber holding the heart-lung. The alterations in volume of this whole system, as shown by the spirometer, *B'*, then correspond entirely to the change in residual air in the lungs. In measuring the oxygen consumption the increase in residual air was substracted from the apparent oxygen consumption to give the true value. While the preparation is in good condition the correction is of the order of 10 per cent and less, but when massive edema occurs the correction becomes very much more considerable. No values for oxygen consumption after edema had set in have been considered in this study for another reason, namely, that edema of the lung impedes the transfer of gases between air and blood. Consequently the oxygen loss from the blood becomes a considerable quantity after edema occurs. Estimation of the oxygen content of the blood in the heart-lung preparation shows no change in periods up to five hours, unless edema occurs, in which case the oxygen content rapidly falls. This finding is in confirmation of Evans.

The glucose in the blood was estimated as reducing substance by the method of Folin-Wu. The free reducing substance in the heart was estimated as by Folin, Trimble and Newman (1927). The glycogen was estimated by a modified Pflüger method, using a small quantity of tissue accurately weighed, employing correspondingly small volumes of fluid in the extraction and the hydrolysis of glycogen to glucose. The Shaffer-Hartman micro method was used for estimating the glucose in the later experiments, the Allihn method in the earlier. Care was exercised in keeping the time interval from the cutting of the heart away from its blood

supply, to the placing of the tissue in the boiling alkali, as short as possible. To facilitate this in the experiments of series D and E, approximately 5 grams of tissue were snipped from the beating ventricle and dropped imme-

TABLE 1

Glycogen content of heart muscle

UNWORKED		WORKED		
Experiment number	Glycogen	Experiment number	Time worked in heart-lung	Glycogen
	per cent		*hours*	*per cent*
B1	0.477	B3	1.25	0.248*
B2	0.740	B5	5.50	0.351
B6	0.661	B10	6.00	0.612
B7	0.375	B12	4.50	0.419
B9	0.384	B15	6.00	0.118*
B11	0.738	B20	5.00	0.528
B13	0.370	B22	4.00	0.370
B14	0.303	B31	7.00	0.120*
B18	0.382	B33	2.00	0.176*
B19	0.450	B35	5.25	0.424
B21	0.424	C5	5.00	0.517
B24	0.458	C11	1.00	0.430
B34	0.559	C15	4.00	0.476
C1	0.759	D2	3.00	0.690
C2	0.610	D5	4.65	0.580
C3	0.482	D7	4.00	0.355
C7	1.030	D11	2.00	0.580
C10	0.482	D13	4.00	0.842
D3	0.910	E12	3.17	0.600
D4	0.325			
D8	0.387			
D10	0.570			
D12	0.540			
E1	0.755			
E2	0.982			
E8	0.421			
Mean	0.560			0.516
Standard deviation	0.165			0.133
Probable error of mean	0.022			0.023

* Terminal edema occurred and results are not included in the mean.

diately into 5 cc. of boiling alkali in a tared centrifuge tube of 50 cc. capacity fitted with a stopper in which a short glass tube was inserted, serving as a reflux condenser. After 15 minutes of heating in the bath the tube was again weighed and the weight of the sample thus determined. Control experi-

ments show that there is a change of two milligrams or less in the weight of the tube fitted with such a condenser during the course of an hour in a water bath.

In our earlier experiments we prepared the heart-lung in the usual way, in which it is impossible to avoid some loss of blood from the system during the course of several hours, and in which it is not possible to correct for changes in the residual air volume. In our later experiments (series C, D, and E) we obviated these difficulties by cutting the heart and lungs entirely free from the chest cavity and placing them in a large closed vessel as indicated in figure 1. In this way we were able to recover quantitatively the blood that was put into the system, and also to measure the dilatation of the lungs. The dilatation of the lungs was measured in only series D and E; in the other series there is a 5 to 10 per cent error from this source in the apparent oxygen consumption.

A. *The glycogen content of hearts with and without work in the heart-lung preparation.* The extent to which the stored carbohydrate in the heart is utilized for the energy needed during the course of several hours' work can be determined by estimating the glycogen content of fresh and worked hearts. The results are shown in the accompanying table 1. Hearts which have worked in the heart-lung preparation for as much as five hours may have as much glycogen in their muscle substance as any of the fresh unworked hearts show. Experiment D13 shows 0.842 per cent glycogen in a heart worked nearly four hours. Apart from any statistical evaluation of the remainder of the data it is obvious that where instances of high normal glycogen content occur in hearts worked for long periods in the isolated heart-lung it is improbable that glycogen was removed as a result of the work. Instances of low glycogen percentage in heart-lung hearts are found to coincide with the occurrence of terminal edema with the associated deficient aeration of the blood. Control experiments show that by stopping the artificial respiration and allowing the heart to beat until virtually all of the oxygen was removed from the blood system, about fifteen minutes, the glycogen content of the heart is reduced to the lowest values that we have ever observed in the heart, in one instance to 0.025 per cent. The fact that even a few minutes of anaerobic activity reduces the heart glycogen to nearly zero, while after six hours of work with no appreciable amounts of carbohydrate other than glycogen remaining in the system the glycogen content has been found as high as 0.612 per cent, suggests that the glycogen serves only in anaerobic metabolism, and that very little, if any of the heart's store of glycogen is used in the oxidative processes occurring in the normal functioning of the heart-lung preparation over the periods we have observed.

The mean value for the glycogen content of normal unworked hearts is 0.560, with a standard deviation of ± 0.165 (in per cent glycogen) and a probable error of the mean (where $P.E._m = 0.6745 \frac{\sigma}{(\sqrt{n})}$) of ± 0.022.

For the worked hearts the mean is seen to be 0.516, the standard deviation ±0.133, and the probable error of the mean ±0.023, all expressed

TABLE 2

Free reducing substance in heart muscle

UNWORKED			WORKED		
Experiment number	Blood glucose	Heart reducing substance as glucose	Experiment number	Blood glucose	Heart reducing substance as glucose
	per cent	*per cent*		*per cent*	*per cent*
B26	0.100	0.150	B31	0.015	0.01
B33	0.099	0.084	B35	0.017	0.01
C12	0.102	0.153	C13	0.080	0.064
E10	0.098	0.087	C15	0.037	0.075
E11		0.109	E12	0.048	0.078
Mean.....	0.100	0.117		0.037	0.047

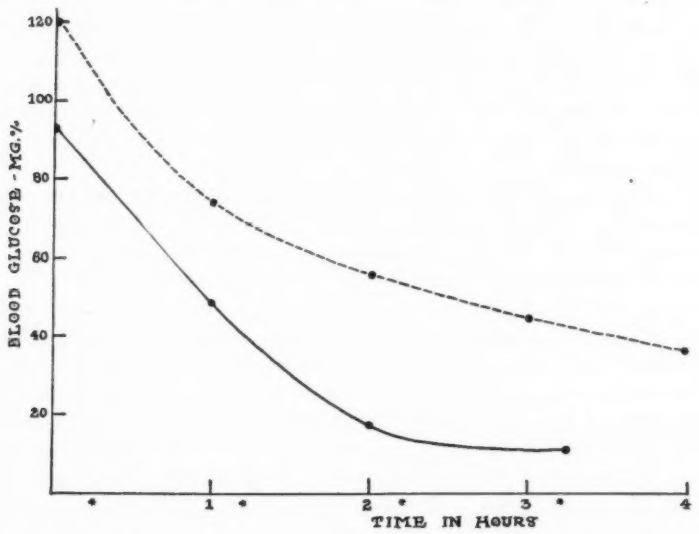

Fig. 2. Rate of disappearance of reducing substance from the blood of the heart-lung.

——— Data from experiment B 31, in which no glucose was added.

---------- Data from experiment C 15, in which 200 mgm. glucose in 5 cc. water were added at intervals indicated by stars on the base line.

in per cent glycogen. The actual deviation of the means of the two series is 0.044 per cent glycogen. It is impossible to state definitely on the basis

of this analysis that there is no significant difference in glycogen content of worked and unworked hearts, but the difference, if any, is small, and negligible in proportion to the oxygen consumed by the heart as indicated later.

B. *The carbohydrate balance in the system.* Figure 2 gives the data of two experiments showing the fall in sugar concentration in the blood of the heart-lung during the course of four hours. We have made studies of glucose disappearance and also the rate of disappearance of reducing substances from the heart muscle. The reducing substances in heart muscle are not entirely glucose, as is evidenced by the fact that they are not entirely fermentable and are partly removed with mercuric nitrate. We found the extraction of reducing substances from the heart a difficult procedure and do not wish to lay stress upon our results, except in so far as they justify us in assuming that, within a reasonable limit of error, the concentration of the reducing substances in the heart varies directly with the blood sugar level. Table 2 presents figures for the analyses of fresh and worked hearts, in comparison with the blood sugar level in the heart at the time. Considerable correspondence is to be seen between the two. This finding is in conformity with the results of Folin, Trimble and Newman (1927). We have, therefore, considered it permissible to assume that the glucose content of the heart and of the lungs, will decrease in the same trend as the sugar in the blood. The relatively small mass of the heart and lungs in proportion to the volume of blood used makes the errors in the analysis of the former smaller.

In table 3 appear the results of observations on glucose disappearance, in comparison with the total metabolism of the heart-lung preparation, as determined by its oxygen consumption. Column 3 gives the value for oxygen consumption in cc./hr., corrected for temperature and pressure, column 4 the total volume of blood and tissue used in each experiment, columns 5 and 6 the findings of the blood sugar estimations at the beginning and end of each experiment, column 7 the calculation of glucose loss per hour from the data in columns 2, 4, 5 and 6, column 8 the oxygen equivalent of the glucose lost, calculating 1 gram of glucose as equal to 744 cc. O_2, column 9 the calculation of the percentage of the total metabolism which can be accounted for by the carbohydrate disappearing, and in column 10 a tabulation of the figures in duplicate analyses of the glycogen content of the hearts after working in the heart-lung preparation for the period specified in column 2.

It will be simpler to understand the basis of our calculations if a specific example of a typical experiment is presented in detail. Experiment D13, a heart-lung preparation which was made to work for four hours will serve as such an example. The animal which was used for the heart-lung weighed 5.5 kgm.; the heart weighed 45 grams; 810 cc. of blood circulated through the preparation; and the total volume of blood and tissue was 975 cc.

The glucose content of the blood initially was 0.112 per cent and at the end, after four hours, it was 0.055 per cent, representing a loss of 57 mgm. per cent. Duplicate glycogen analyses showed 0.841 per cent in the heart at the end of the experiment. This is a high normal glycogen content and it may be assumed that there has been no significant loss of glycogen from the heart during the period. The loss of glucose from the system amounts to 0.557 gram during a period of four hours. Four hundred and fifteen

TABLE 3

Carbohydrate in relation to total metabolism of heart

EXPERIMENT NUMBER	HOURS	O_2 USE	VOLUME OF BLOOD AND TISSUE	GLUCOSE CONCENTRATION		GLUCOSE LOSS	O_2 EQUIVALENT OF GLUCOSE USED	PER CENT OF O_2 CONSUMPTION DUE TO CARBOHYDRATE	FINAL GLYCOGEN CONTENT OF THE HEART
				Beginning	Ending				
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)
		cc. per hour	*cc.*	*per cent*	*per cent*	*mgm. per hour*	*cc. per hour*		*per cent*
B35	5.00	302	800	0.129	0.017	195.2	145.5	48.2	0.424
C5	3.00	460	800	0.078	0.022	146.0	108.6	23.6	0.517
C9	1.75	322	600	0.108	0.038	240.0	178.6	55.4	Lost
C13	2.00	515	800	0.102	0.080	288.0*	213.2	41.3	Lost
C15	3.75	516	700	0.120	0.037	368.4†	273.7	53.0	0.476
D2	2.33	402	750	0.070	0.015	177.0	131.7	30.1	0.690
D5	4.00	668	1,250	0.102	0.035‡	209.4	155.7	23.3	0.576
D7	3.17	310	975	0.091	0.022	212.2	157.8	50.9	0.355
D9	1.50	257	1,000	0.129	0.103	173.3	129.9	58.6	Lost
D13	4.00	530	975	0.112	0.055§	138.9	103.3	19.4	0.842
E12	2.57	472	1,700	0.067¶	0.046	138.9	103.3	21.2	0.600

* In the period of two hours a total of 400 mgm. of glucose was added to this preparation.

† In the period of three and three-fourths hours 800 mgm. glucose were added.

‡ Final glucose concentration in the circulating blood was 0.024 per cent, the figure given is for the mixed blood from the circulating system itself and that which had leaked into the container.

§ Final glucose in circulating blood was 0.036 per cent.

¶ Low beginning glucose concentration is due to the fact that the preparation had run for an hour before measurements could be begun.

cubic centimeters of oxygen would burn that amount of glucose. Actually we observed that the average oxygen consumption was 530 cc. per hour or 2120 cc. in the whole period. We infer that the heart was using glucose for less than 20 per cent of its total energy.

In general the shorter periods show the use of larger percentages of carbohydrate in the total metabolism. This is probably due to the fact that in the earlier part of the experiment the glucose content of the blood as we have used it is higher. In two experiments, number C13 and C15, glucose was

added to the extent of 400 and 800 mgm. respectively. In these experiments the proportion of O_2 consumption due to carbohydrate burning is somewhat larger, as we should expect, but still only 50 per cent.

It is well to point out that in a case like experiment D5 even if all of the carbohydrate which was in the system at the commencement of the heart-lung preparation had been burned, there would still have been 45.9 per cent of the total metabolism unaccounted for. The heart weighed approximately 60 grams, and assuming that it contained 1.00 per cent glycogen, a value of the highest order found, it might have contained 0.600 gram of glycogen. This corresponds to 495.5 cc. of oxygen. The total free glucose present in the system was 1.275 gram, equivalent to 948.6 cc. of oxygen. The combined oxygen equivalent is 1444.1 cc. The oxygen consumption during this time was 2672 cc., leaving an excess of 1228 cc. over that used in burning carbohydrate. Similar calculations could be made upon several other experiments, as C5, D2, D13, and E12.

In our experiments we have not attempted to measure CO_2 production, because of inherent practical difficulties. The experiments of Bayliss, Müller and Starling (1928) provide, however, material for comparison with our own results. These investigators have been able to measure the oxygen and carbon dioxide exchange of the heart-lung preparation under such conditions that the carbon dioxide output probably represents a real metabolic CO_2 production. Former studies on the R. Q. of the heart-lung have been in error because they were carried out under conditions which did not prevent the progressive acapnia of the preparation. Bayliss, Müller and Starling maintained a constant alveolar CO_2 tension and avoided a reduction in the CO_2 in the blood. Their CO_2 excretion probably represents very nearly the metabolic CO_2 production of the preparation. Unfortunately their published observations are on cases where the blood sugar concentration had been enormously raised by addition of glucose. Consequently none of their observations are closely comparable with our own, where the blood sugar was usually at or below 0.100 per cent. In two experiments they present blood sugar values along with metabolic data. In one they find an R. Q. of 0.82 while the blood sugar is 0.55 per cent. Evidently even in the presence of the relatively enormous amounts of glucose the carbohydrate metabolism is less than 40 per cent of the total. In this same experiment increasing the blood sugar concentration to 2.4 per cent raised the R. Q. to about 0.87. In another experiment, with the blood sugar at 0.2 per cent, the R. Q. was about 0.84. With the addition of insulin, and the blood glucose raised to 0.5 per cent, the R. Q. became steady at 0.93. The amount of glucose lost by our heart-lung preparation would lead us to expect respiratory quotients in the range between 0.78 and 0.87.

C. *The blood sugar in relation to the glycogen content of the heart.* In

table 3, columns 6 and 10, are given the analyses of glycogen in the heart and of glucose in the blood at the end of the experiment with the heart-lung. The glycogen in the heart bears no relation to the glucose in the blood circulating in the heart at the time the sample for glycogen estimation was obtained. In experiment D11, for example, the concentration of reducing substance in the blood, reckoned as glucose, was 0.015 per cent while the glycogen content of the heart was 0.690 per cent. Practically the whole of this residual reducing substance is non-fermentable material, and therefore presumably not glucose. Evidently heart glycogen is not a reserve upon which falling blood sugar may call, nor is it a supply of material for the oxidative metabolism. Glycogen appears then to be a part of the machine of the muscle, and does not under these conditions contribute to the fuel for operating the machine.

In this series of experiments we have not followed the lactic acid in the circulating blood except in one experiment. The work of Anrep and Cannan (1925) and Tsuji (1916) has demonstrated that under the conditions of our experiments the lactic acid in the blood of the heart-lung does not fall, but rather increases in amount.

SUMMARY

1. It is found that hearts worked in the heart-lung preparation for periods up to six hours contain the same quantities of glycogen as are found in normal, unworked hearts. The mean value for a series of worked hearts does not vary significantly from the mean of normal hearts. It is improbable that work in the isolated heart-lung causes an appreciable lowering of heart glycogen.

2. It is found that the free reducing substance in the heart muscle diminishes coincidently with the reducing substance in the blood supplying the heart of the heart-lung preparation.

3. The free reducing substance in the heart may diminish to such a figure that it is probably nearly a zero value as far as glucose is concerned, while the glycogen content remains at a value similar to values obtained in unworked hearts.

4. Comparison of the oxygen consumption with the glucose utilization of the heart-lung preparation shows that in no instance does the heart-lung preparation, under the conditions of our experiments, use heart glycogen or blood glucose for more than 60 per cent of its metabolism, and in some instances these carbohydrates form less than 25 per cent of the fuel.

5. There is a rough agreement between carbohydrate burning as we observed it, and as others have calculated it from the respiratory quotient.

6. All of the carbohydrate in the system at the beginning of an ex-

periment would not account for the total metabolism of the heart-lung over a period of five hours.

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A CINEMATOGRAPHIC STUDY OF SYSTOLIC AND DIASTOLIC HEART SIZE WITH SPECIAL REFERENCE TO THE EFFECTS OF ANOXEMIA

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Received for publication May 16, 1930

Although the circulation of man and of animals under decreased oxygen tension has been the object of frequent researches, a difference of opinion still exists upon the question whether anoxemia exerts a preliminary stimulating effect upon the heart or whether it has only a depressant action. The conflicting literature as to whether the systolic discharge and minute output increase during progressive anoxemia was reviewed by Sands and DeGraff (1925). On the basis of dynamic studies, they themselves conclude that the systolic discharge is not affected by a mild degree of anoxemia, is increased by anoxemia of moderate grade and is not essentially decreased until the circulating crisis suddenly occurs.

The occurrence of such beneficial effects of moderate anoxemia is only supported in part by studies of volume curves. Mathison (1910) found that the diastolic size and systolic discharge decrease when nitrogen alone is administered. Garisch and Wastl (1926) however observed an increase in systolic discharge in cardiometric experiments. Gremels and Starling (1906) found that the isolated heart is unaffected by low oxygen tension until it becomes extreme; then great dilatation and diminished output occur. They attributed the early stimulating effects observed by others to effects mediated through the central nervous system. That such actions cannot be ignored is also indicated by the observations of Greene, Payne and Siddle (1925).

The question at once arises as to the validity of cardiometric studies, in detecting changes in ventricular volume. Properly used it is a valuable and accurate method for determining relative changes in ventricular volume during successive moments of systole and diastole. When the method is used to compare the systolic discharges and diastolic sizes of separate beats its accuracy is conditioned by the character of the experiments. When the diastolic size does not vary greatly, when special pre-

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cautions are taken to maintain a constant relation of the rubber diaphragm to the ventricular base and when the experimental variations in the heart occur within a reasonable span of time, the method is reliable qualitatively and perhaps roughly quantitatively. There are many experimental studies, however, in which maintenance of such conditions becomes difficult or quite impossible. For instance, experiments in which the effects of anoxemia, hypercapnia, asphyxia, etc., were studied over long periods of time showed that changes in the circumference of the ventricular base and unusual movements of respiration rendered it impossible to control the placement of the heart in the cardiometer and to maintain a proper fitting between the ventricle and the opening in the rubber diaphragm. Such a method of registration is therefore unsuited for experiments extending over long intervals. When all of these factors are taken into consideration it becomes obvious that under such conditions changes in systolic and diastolic size, as well as variations in systolic output lose their quantitative and in many cases their qualitative meaning.

The cinematographic method of studying changes in heart size during anoxemia was employed by Takeuchi (1925). He concluded that anoxemia of mild degree always causes an increase in diastolic size and inferred according to Starling's law that this must cause an increase in systolic discharge. But Takeuchi did not avail himself of the maximum possibilities of the method and made no experimental tests of its reliability. Therefore, following a suggestion of Dr. C. J. Wiggers, I investigated the possibility of studying volume changes by means of a cinematographic method, not, be it understood, with the intention of replacing the cardiometric method in general but with the idea of supplementing it in experiments in which its application becomes difficult and its interpretation questionable.

THE CINEMATOGRAPHIC METHOD. The cinematographic method consisted in exposing the heart of a dog (anesthetized with barbital) and supporting it in a cradle of pericardium made by stitching it to the chest wall. Photography was carried out by means of a Bell-Howell camera equipped with an F. 2.7 Zeiss lens and utilizing a 16 mm. panchromatic film. This method proved particularly satisfactory on account of the trade arrangements for development of the film. The movie camera was placed at a distance of 1 foot from the anterior surface of the heart. This yielded a picture which approximately filled the entire width of film. The animal board was slightly tilted, so that the plane of the heart was parallel to the surface of the film in the camera. The heart was illuminated by three 1000 watt Mazda lamps equipped with reflectors, and after each series of pictures a number, written with chalk on a small blackboard, was photographed, thus enabling identification of records when simultaneous optical records of aortic pressure were made. After development and

conversion into a positive film the individual frames were projected and magnified 2.5 times on a paper-screen (the surface area magnified approximately 6×). The circumference of each projected picture was drawn with a pencil and subsequently measured by means of a planimeter. Figure 1 shows a series of such overlapping drawings, representing the changes in size during one heart cycle.

Physical checks of the method: Constancy of camera speed and exposure intervals. As the moving picture camera used depended on a spring drive, it was of first importance to control the regularity of its motion. The camera was set at its rating of 32 pictures per second which theoretically gives one picture nearly every 0.03 second. Tests were made as follows: An electric lamp was arranged in the movie camera and the camera

TABLE 1

Durations

NUMBER OF PICTURE FROM START	DURATION OF EXPOSURES	DURATION OF CLOSURES
	second	*second*
1	0.07	0.025
2	0.03	0.017
3	0.025	0.013
4	0.02	0.01
5	0.02	0.01
15	0.02	0.01
30	0.02	0.01 at about 1 second
45	0.02	0.01
60	0.02	0.01 at about 2 seconds
75	0.02	0.01
450	0.02	0.01 at about 15 seconds

was set before a photokymograph. A tuning fork (period 0.02 sec.), vibrating in front of the slot, registered its vibrations during the intervals that light passed through the shutter of the movie camera while it was open and not during the periods that it remained closed. Examination of many such records taken from the moment the movie camera was started until it had fully run down, gave the following information: 1. The first 2 to 4 pictures taken are exposed too long owing to the inertia of the moving parts. 2. Afterwards and up to a time the camera has run for 15 seconds the intervals of exposure were constantly 0.02 second and the intervals of closure a trifle more than 0.01 second. 3. After 15 seconds the camera slows down, the times, both of exposure and of closure, increasing successively in trifling amount. These facts are shown by the data in table 1.

In view of these observations we re-wound the camera previous to each registration, limited the period of exposure to 5 or 6 seconds and discarded from use the first half-dozen pictures of any series studied.

Optical errors due to movement of the heart during systole and diastole. According to elementary optical principles, the size of a photographic image is altered by changing the distance between the object and the camera lens. Since the rotation of the heart about a transverse axis and the forward movement of the apex brings the whole heart somewhat nearer to the camera lens during systole it was anticipated that the areas photographed during systole would be somewhat too large when compared with those representing diastole. The magnitude of the error proved to be small however. Observation, measurement by various mechanical devices, as well as direct registration of the forward movement on a drum (fig. 2), showed that the transverse plane of the heart (i.e., the boundaries of

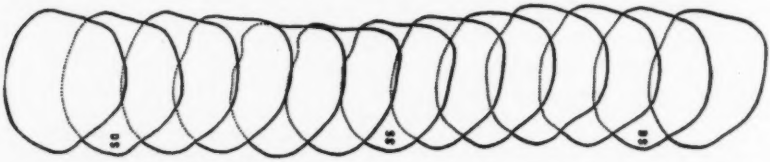

Fig. 1. Greatly reduced series of retraced ventricular areas during one heart cycle. Reproduced to illustrate nature of areas measured by planimeter. *SS*, minimum systolic size; *DS*, maximum diastolic size; entire series taken in about ½ second.

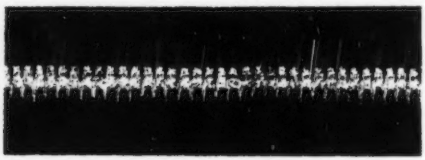

Fig. 2. Drum tracing (actual size) showing actual changes in forward movement of heart during systole and with long inflation. Forward movement indicated by downward movement in tracing.

ventricles) moved forward not more than 6 mm. during systole. Knowing the distances, the focal lengths of the lenses used and the magnification ultimately attained in projection, the error incurred in the large record was determined by mathematical processes. It was also determined experimentally by aligning at correct distances on an optical axis, the camera and projector lenses and a luminous circle of known area. By moving the luminous circle forward a distance of 6 mm. the change in the area of the projected circle could be directly determined. Both methods were in agreement that the systolic area is about 8 per cent too large when compared with the diastolic area. In other words the full difference between systolic and diastolic size is 8 per cent short of being fully recorded.

In addition the heart rotates about its long axis during systole, exposing

more of the left and less of the right ventricular surface. Any error of the frontal area thus occasioned must be algebraically added. This error is difficult to evaluate mathematically, hence I resorted to experimental tests. The heart of a dog recently dead was ligated, cut out and placed in different positions corresponding to the maximum rotation observed in living animals. Figure 3a, b shows two prints in reduced size of a heart which had a total volume of 60 cc. Measurement of the areas (3705 mm.^{2} and 3735 mm.^{2} respectively) shows an insignificant error due to rotation. We feel therefore justified in concluding that the error due to change of position during systole and diastole is not greater than 10 per cent.

Optical photographic errors due to forward movement of entire heart during respiration. Suspension of the heart in a pericardial cradle itself reduces the effect that lung inflation and deflation have on the position of the heart. Indeed this effect is often not noticeable or recordable provided artificial respiration is kept at a minimum. Moreover the pictures were always taken at the end of the phase of expiration so that this factor could not enter. Nevertheless we deemed it advisable to determine the differences in area due to displacement of the heart by lung inflation and deflation. Figure 3, c, d shows two pictures of a heart shortly after death of the animal, c, in complete inflation, d, in complete deflation of the lungs. Even under these extreme conditions of lung inflation and deflation the changes in size of the optical projection areas were insignificant, measuring 3870 mm.^{2} in the deflated and 3778 mm.^{2} in the inflated state of the lungs. We conclude that the effect of lung inflation is negligible. But, as already mentioned, the pictures were taken only at the end of the deflation, by which device the disturbances resulting from respiration were avoided with certainty.

Errors due to re-drawing and measurement. It is obvious that precision in the use of the planimeter and in tracing the projected heart areas must be acquired and the personal equations of those concerned must be established. For this reason all the outlines were drawn by the same person (Str.) and the planimeter measurements were all made by Mr. Shaw. Surface area measurements were all checked by determining first the total area, and then the sum of the left and right ventricular areas, measured individually. Whenever differences exceeded 30 mm.^{2} in areas of 20,000 or 30,000 mm.^{2} or more, the curves were measured a third time.

The relation between surface area and ventricular volume. For geometrically similar forms the relation between volume and area is given by the expression: Volume $= C \times A^{3/2}$, where C is a constant. For small changes in size the deviation from proportionality is very often insignificant. To get a notion how far this may hold good for the heart I tried first what Bardeen (1918), Skavlem (1922) and Eyster (1928) had done before, comparing ventricular volumes and surface area in dead hearts.

My chief interest lay, however, in studying volume changes in the same heart, which I carried through in the following way. Immediately after death, the large vessels at the cardiac base were ligated by a stout mass

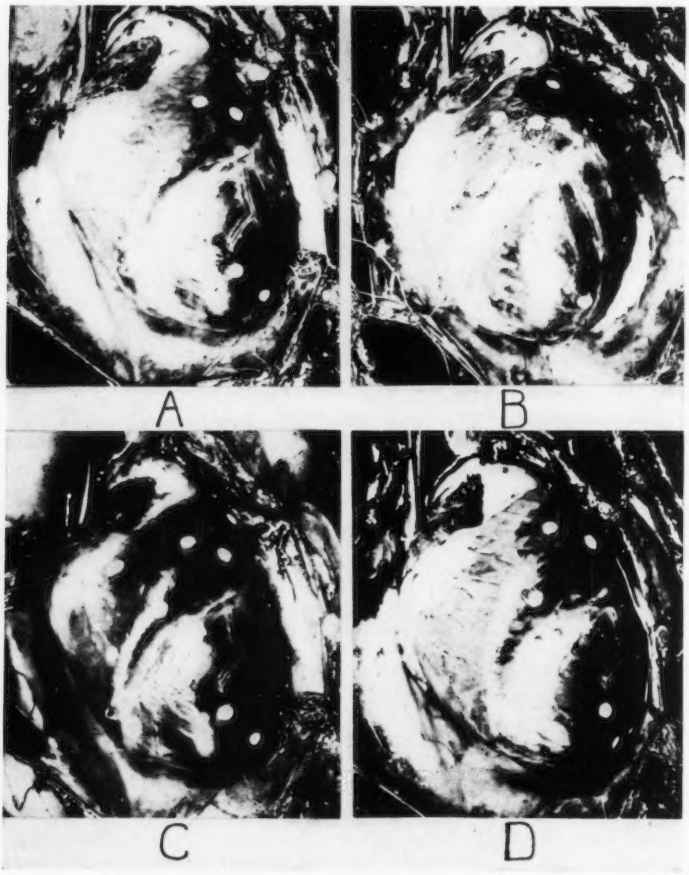

Fig. 3. Four enlarged photographs showing nature of heart films and areas included by their boundaries. Discussion in text.

ligature or large clamp, large cannulae were inserted into the right and left auricle and the chambers washed clean of blood. The ventricles were then filled with saline at a pressure of 50 mm. saline, i.e., approximately the normal venous inflow pressure. Then small amounts of saline solution

were introduced by injection until the heart could accommodate no more fluid and photos taken after every 5 or 10 cc. injected.

The data, incorporated in the plot of figure 4, show that a good proptionality exists between the surface areas enlarged 6 × and the ventricular volumes as long as the increasing distention of the heart did not advance the heart too much toward the camera.

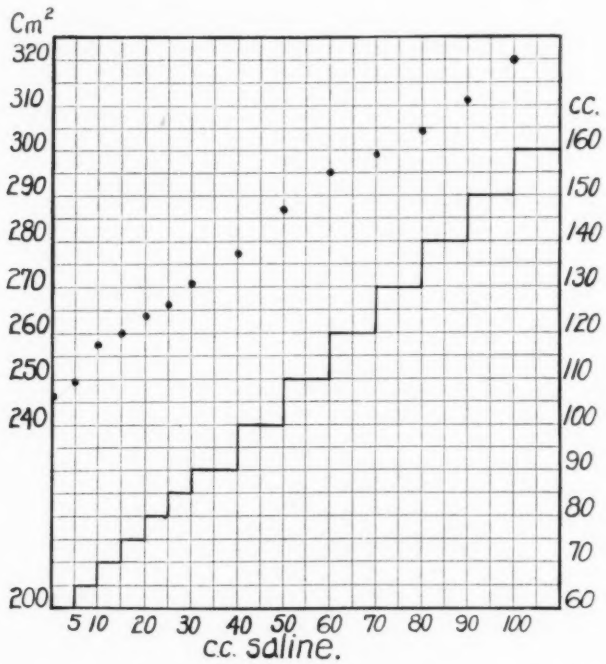

Fig. 4. Comparison of changes in volume and in photographed areas (6X) after introduction of successive increments of fluid into a ventricle with an initial vol. = 60 cc. Dots represent changes in areas in cm.²

Such experiments validate the employment of surface area measurements only so far as changes in diastolic size are concerned. They give no reliable information as to proportionalities during different stages of systole. It occurred to us that the existence of such a proportionality, if present, would be reasonably established by plotting the changes in surface areas in successive pictures in the form of a graph. Only if definite proportionality was maintained and only so, would the curve resemble a normal volume curve of the ventricle.

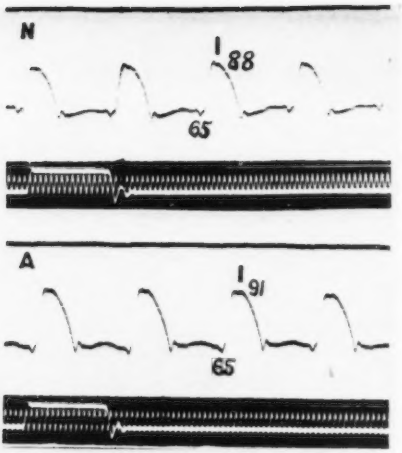

Fig.-5-a.

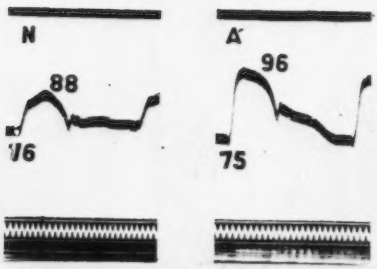

Fig.-6-a.

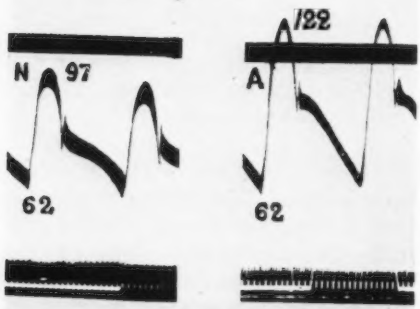

Fig.-7-a.

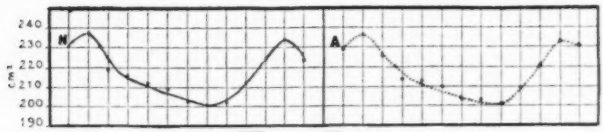

Fig. 5-b.

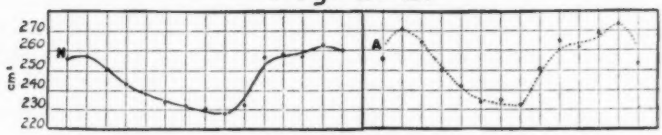

Fig. 6-b.

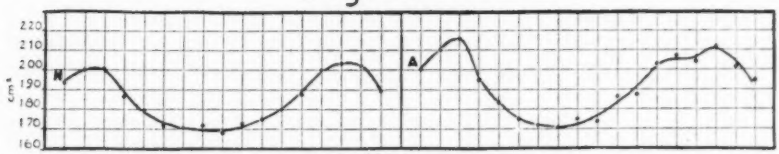

Fig. 7-b.

Fig. 5 b. Plot of successive ventricular areas showing changes during systole and diastole and also largest and smallest sizes during a cycle. *N*, ventilation with normal air; *A*, ventilation with gas mixture having lower oxygen content. Abscissae, 0.031 second; ordinates in cm.² From experiment I. Discussion and comparison with figure 5 a in text.

Fig. 6 b. Same as 5 b but from experiment II. Discussion and comparison with 6 a in text.

Fig. 7 b. Same as 5 b but from experiment III. Discussion and comparison with 7 a in text.

Since the cinematographic method yielded a succession of cardiac sizes at intervals of 0.031 second, I adopted this as the value of the abscissal units, plotting the surface area values of the six times enlargement of actual heart areas as ordinates. The nature of the plots is sufficiently illustrated by the graphs of figures 5b, 6b and 7b. The general resemb-

Fig. 5 a. Aortic pressure curves recorded with calibrated manometer in experiment I, when dog's lungs were ventilated with normal air, *N*, and with a lower O_2 gas mixture, *A*. Tuning fork = 0.02 second. Time signal correlated with movie pictures. Systolic and diastolic pressure values marked directly on records. Discussion and comparison with figure 5 b in text.

Fig. 6 a. Same as figure 5 a; from experiment II. Discussion and comparison with figure 6 a in text.

Fig. 7 a. Same as figure 5 a; from experiment III. Discussion and comparison with figure 7 a in text.

lance to volume curves obtained by oncometric methods is obvious. In these instances the heart was beating rapidly but, it may be added, that in other graphs obtained during vagus stimulation, the prolonged period of diastole and even the changes during auricular systole were clearly indicated.

Other experimental procedures. While I felt that the method was sufficiently accurate to study the changes in systolic and diastolic size during anoxemia and, by their difference, changes in the systolic discharge as well, it was felt desirable to check the procedure by recording simultaneously also the variation in aortic pulse pressure. This was done by use of a Wiggers manometer, calibrated with respect to a base line previous to each experiment. In order to identify the different heart beats in the movie picture for comparison with the corresponding ones in the pressure curve, two signals in the same electric circuit were arranged before the photokymograph and the movie camera (near to the heart). This signal could be observed in the pressure curve (fig. 5 a) and in the movie pictures as well.

In order to separate the cardiac effects of anoxemia which are *primary* from those which are *secondary*, in the sense that these terms have been defined by Wiggers (1927, 1928) it was necessary to study the effects in "controlled circulation experiments," i.e., while the heart rate is kept constant by rhythmic artificial stimulation of the right auricle (after clamping the S-A node to reduce the natural rate) and while changes in arterial pressure and venous pressure are compensated. This was done by methods which have been adequately described by Wiggers (1928, 1929).

Anoxemia was produced in the following manner: During the course of the experiment *respiration* was accomplished by a motor driven pump. The discharge of the pump could be changed between 250 to 400 cc. of air. The rate of respiration was 12 to 14 per minute. The pump was also connected with an 80 L. tank, in which by dilution with nitrogen the oxygen content was decreased to between 10 and 5 volumes per cent. By turning a stopcock, normal air or the gas mixture could be pumped alternately into the dog's lungs.

General analysis of results. The general character of the results is illustrated by the three sets of aortic pressure curves reproduced as figures 5a, 6a and 7a and the heart size curves from identical beats reproduced as figures 5b, 6b and 7b. The detailed data of these experiments are incorporated in table 2. A few general points may be brought out before we proceed to their detailed analysis.

In 10 experiments in which the heart rate alone was kept constant the results were the same as those observed by Sands and De Graff (1925) in which the heart rate altered. During the first stages of anoxemia the diastolic aortic pressure rose slightly (20 mm. Hg and more). This was

accompanied by a considerably greater rise of the systolic pressure. Therefore we have an increase of pulse pressure which often reached 90 per cent or more. The venous pressure was sometimes slightly lowered, sometimes almost unchanged. After 10 minutes and more when air of less than 8 per cent of oxygen was given I observed a rapid decrease of systolic pressure

TABLE 2

Direct effects of anoxemia on dynamics of circulation and on systolic and diastolic size of heart

	DYNAMICS OF CIRCULATION							SIZE OF HEART			
Experiment	O_2 content	Cycle	Venous pressure	Diastolic aortic pressure	Systolic aortic pressure	Pulse pressure	Increase of pulse pressure	Systolic size	Diastolic size	Difference between diastolic and systolic size	Ds/Ss ratio
	volume per cent	*second*	*mm. saline*	*mm. Hg*	*mm. Hg*	*mm. Hg*	*mm. Hg*	*cm³.*	*cm³.*	*cm³.*	
I-N	21	0.35	30	65	88	23		201.0	238.4	37.4	1.186
I-A	After 10 minutes 10.5% O_2	0.35	30	65	91	26	3 (= 13%)	201.3	236.9	35.6	1.177
II-N	21	0.43	20–25	76	88	12		168.6	201.4	32.8	1.194
II-A	After 10 minutes 8% O_2	0.43	20–25	75	96	21	9 (= 92%)	171.0	216.0	45.0	1.263
III-N	21	0.35	45–50	62	97	35		227.9	257.4	29.5	1.129
III-A	After 3 minutes 5.3% O_2	0.35	45–50	62	122	60	25 (= 71%)	232.5	271.2	38.7	1.166
IV-N	21	0.47	23–28	73	103	30		176.0	203.6	27.6	1.157
IV-A_1	After 5 minutes 7% O_2	0.47	23–28	73–74	111	37.5	25	181.0	212.4	31.4	1.173
IV-A_2	After 9 minutes 7% O_2	0.47	23–28	74	110	36	20	178.2	209.9	31.7	1.172

and a slow falling of the diastolic pressure, while a marked increase of venous pressure occurred (crisis). The pulse pressure decreased more and more and after a few minutes complete circulatory collapse set in unless normal air was given to the dog.

Detailed analysis of typical experiments with controlled heart, diastolic aortic pressure and venous pressure. As I have stated, the diastolic aortic pressure

rises during anoxemia. In order to obtain curves with equal diastolic pressure from the animal breathing normal air as well as air with decreased oxygen content I chose the following way: By obstructing the aorta abdominalis of the dog breathing normal air I took a series of records at different diastolic pressures. Then after releasing the compression, the prepared gas mixture was given from the tank. During anoxemia, the diastolic pressure increased again as already described and thus it was possible to get curves with equal arterial resistance from the dog under both conditions. In a series of 10 experiments I succeeded in obtaining a number of records the most characteristic of which I would like to describe.

Experiment I was performed on an anesthetized dog with vagi intact. Heart rate, arterial and venous pressures were held constant, as is shown by data in table 2. Figure 5a shows the optical pressure pulses while breathing atmospheric air, *N*, and after breathing a 10.5 per cent oxygen-nitrogen mixture for 10 minutes *A*. The measured systolic and diastolic pressures are indicated directly on the record. It will be noted that the systolic pressure rose from 88 to 91 mm. Hg, hence the pulse pressure increased only by an insignificant amount. This indicates that the systolic discharge was not altered. If we compare with this the data found for the heart size obtained from the movie pictures (fig. 5b and table 2) we also find no noteworthy change either in systolic or diastolic size and consequently not in the $\frac{\text{diastolic}}{\text{systolic}}$ ratio.[2] According to this experiment neither the pulse pressure nor heart size measurements indicate any noteworthy effect upon the heart itself during a slight degree of anoxemia. I publish the data and figures of just this experiment because it demonstrates the accuracy of the cinematographic method.

In experiment II the vagi nerves were left intact. Figure 6a shows two segments of aortic pressure curves, one, *N*, taken while the animal breathed normal air and the second, *A*, after breathing an 8 per cent O_2 mixture. As is revealed by the data in table 2, heart rate, venous pressure and diastolic pressures remained practically constant. The systolic pressures rose markedly, thereby increasing the pulse pressure about 92 per cent. Under the conditions of the experiment this could only signify a pronounced increase in systolic discharge.

The curves and measurements derived from moving pictures for the same heart beat are shown in figure 6b and in table 3. We found an increase in systolic size of 1.4 per cent and in diastolic size of 7.2 per cent. The difference between diastolic and systolic size of the enlarged heart for

[2] This value signifies the size of the heart in diastole, if the systolic size is set equal to 1.0. I find it useful to calculate this ratio, since it gives a good insight into possible conclusions which could be made from relative changes of systolic and diastolic size.

N. is 32.8 cm². and for A, 45.0 cm². The $\frac{\text{diastolic}}{\text{systolic}}$ size ratios amount to 1.194 respectively and 1.263 respectively. These observations confirm the pressure curves in showing that moderate anoxemia increases the systolic discharge in "controlled circulation" experiments.

Similar results were obtained in experiment III in which both vagi were cut. Figure 7a shows the optical pressure curves before, *N*, and after breathing a 5.3 per cent oxygen mixture, *A*. After 3 minutes the pulse pressure increased from 35 to 60 mm. Hg or 25 mm. As is shown in this figure and also in the data of table 2 this is due entirely to an increase in systolic pressure. Since the venous pressure and heart cycle were also

TABLE 3

EXPERIMENT NUMBER	DEGREE OF ANOXEMIA	INCREASE OF PULSE PRESSURE	INCREASE OF SYSTOLIC SIZE	INCREASE OF DIASTOLIC SIZE	D_S/S_S RATIO FOR N (NORMAL AIR) AND A (ANOXEMIA)
		per cent	*per cent*	*per cent*	
1	10.5% O_2 after 10 minutes	13	0.10	1.5 (decrease)	N: 1.186 A: 1.177
2	8.0% O_2 after 10 minutes	92	1.4	7.2	N: 1.194 A: 1.263
3	5.3% O_2 after 3 minutes	71	2.0	3.8	N: 1.129 A: 1.166
4 a	7.0% O_2 After 5 minutes	25	2.8	4.3	N: 1.157 A: 1.173
4 a_2	After 9 minutes	20	1.3	3.8	N: 1.157 A: 1.172

kept constant, this can only signify an augmentation of systolic discharge due to a direct effect of anoxemia.

This is confirmed by the study of heart size areas. As shown in figure 7b and in table 2 the systolic size increased only 2 per cent, whereas the diastolic area increased 3.8 per cent. The difference between diastolic and systolic size increased from 29.5 to 38.7 cm². The D/S ratio was 1.129 (N) and 1.166 (A). The data of experiment 4 are recorded in table 4. They are similar in every respect and lead to identical conclusions.

CONCLUSIONS. In order to get a more comprehensive view of the results obtained in these experiments they are gathered together in a single table (table 3).

The following facts appear to be established: 1. Excluding experiment 1, during which a very mild form of anoxemia existed, we find that the pulse

pressure increases on an average 50 per cent. This indicates that the systolic output of the heart increases and since the heart rate is kept constant the minute volume is larger. The increased difference between systolic and diastolic areas shown directly in the graphs of figures 5b, 6b and 7b and conveniently expressed by the D/S ratio, substantiates this conclusion. The value of D/S averaged $\frac{11}{10}$ in normally breathing dogs and $\frac{13}{10}$ in dogs in various stages of anoxemia.

2. In all experiments in which an increased systolic discharge occurred, the heart rate, venous pressure and arterial diastolic pressure remained constant, thus proving that oxygen deficiency has a direct stimulating effect on the ventricles.

3. Since the diastolic size increased between 3.8 to 4.3 per cent in all experiments in which an increased discharge was noted and as this occurred in spite of the fact that venous pressures remained unaltered we have proof that oxygen deficiency causes an increased initial length by its direct action on the heart. According to Starling's law this probably accounts for the increased systolic discharge.

4. All of these direct effects which may be designated as "stimulating effects of anoxemia" do not appear until the oxygen content of the inspired air falls below 10 per cent. They disappear again after the "critical stage" is reached. The pulse pressure decreases more and more, both systolic and diastolic sizes increase and the D/S ratio approaches 1.0. Since it becomes almost impossible to maintain venous pressures and arterial resistance constant, this phase of circulatory dynamics does not lend itself to careful study in "controlled circulation experiments."

SUMMARY

The questions, whether the systolic discharge and minute volume are increased by anoxemia of limited degree and if so whether this is due to a primary effect on the ventricle or to secondary changes in venous pressure, arterial heart rate were reinvestigated.

All studies were made on anesthetized dogs with "controlled circulations," i.e., the heart rate, arterial diastolic pressure and venous pressure were kept constant.

A cinematographic method for recording changes in heart size during successive moments of the heart cycles was developed and its reliability was determined by physical tests.

By enlarging the individual forms of pictures so obtained, redrawing and measuring their surface areas, changes in size during consecutive intervals of about 0.03 second could be plotted.

In this way the effect of anoxemia on systolic and diastolic size could be studied.

The effect on systolic discharge could also be estimated by their differ-

ence and the changes noted, compared with alterations in arterial pulse pressure recorded by optical manometers.

The results showed that anoxemia produced by gas mixtures containing less than 10 per cent of oxygen produce the following sequential changes by direct action on the heart. *a*. An increased diastolic volume, independent of changes in venous pressure (increased initial length). *b*. An increased systolic discharge. *c*. Increased systolic pressure and pulse pressure, when diastolic pressure was kept constant.

That any considerable lowering of oxygen tension of blood is compensated for by an increased minute volume can no longer be questioned, for in addition to the secondary effects of anoxemia (such as increase in heart rate), the systolic discharge is also increased by a direct cardiac action.

It may incidentally be added that the stimulating effect of asphyxia on the heart appears to be exclusively due to the associated anoxemia, for Wiggers (1929) recently demonstrated that no direct effect of hypercapnia can be demonstrated in similar experiments.

In conclusion, I wish to express my appreciation and gratitude to Prof. C. J. Wiggers for his assistance in this work and for the facilities kindly offered in his department.

My thanks are also due to Miss A. Maltby, Mr. Shaw and Mr. Theisen for valuable help.

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THE RELATION OF GONADAL CONDITION TO ERYTHROCYTE NUMBER IN FOWLS[1]

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Received for publication May 24, 1930

The occurrence of a distinct sex difference in the number of red corpuscles in fowls was recorded by J. Blacher in 1926. The author found that in the male the erythrocyte count averaged 3,772,000, in the female 2,870,000.

In the gonadectomised bird of either sex the number of erythrocytes was similar to that in the female. From these observations Blacher concluded that, in the male, castration resulted in a diminution of the number of red corpuscles, while in the ovariotomised female the original level was retained.

Chaudhuri (1926) in Crew's laboratory substantiated these findings, and extended his own observations to include 15 immature birds of either sex and 14 hens, which having functioned as females in their earlier history, later underwent transformation into cocks. The sexually abnormal birds exhibited an erythrocyte count closely approximating the male number. In the immature birds no sex differences were noted: the number of red corpuscles here being rather intermediate between the adult male and female. This appears from the comparison given below which is compiled from the averages given in Chaudhuri's paper.

ADULT MALE	ADULT FEMALE	IMMATURE MALE	IMMATURE FEMALE
4,560,000	3,127,000	3,603,000	3,656,000

During an extensive series of observations on birds of both sexes carried out as part of the program on the biology of sex now being continued under the direction of Prof. F. R. Lillie at the Whitman Laboratory of Experimental Zoölogy, systematic records were kept of the erythrocyte count of many birds from the date of hatching to maturity. Erythrocyte counts were also made in poulards having an hypertrophied right gonad, in bilaterally ovariotomised females and in capons.

[1] The expenses of this investigation were supported in part by the Committee for Research in Problems of Sex of the National Research Council: grant administered by F. R. Lillie.

I. THE NORMAL SERIES: The number of red corpuscles per cubic millimeter was determined on a total of 80 birds, 40 females and 40 males. The group was obtained as one day chicks from the dealer supplying all the bird material used in this laboratory and kept in adjacent pens under uniform conditions. With the exception of ♂♂ 02, 04 and ♀♀ 01, 03, 05, and 07 all the birds were hatched the same day, April 30, 1928.

Blood was obtained from the wing vein. Trenner automatic pipettes were used and the Levy-Hauser counting chamber with improved Neu-

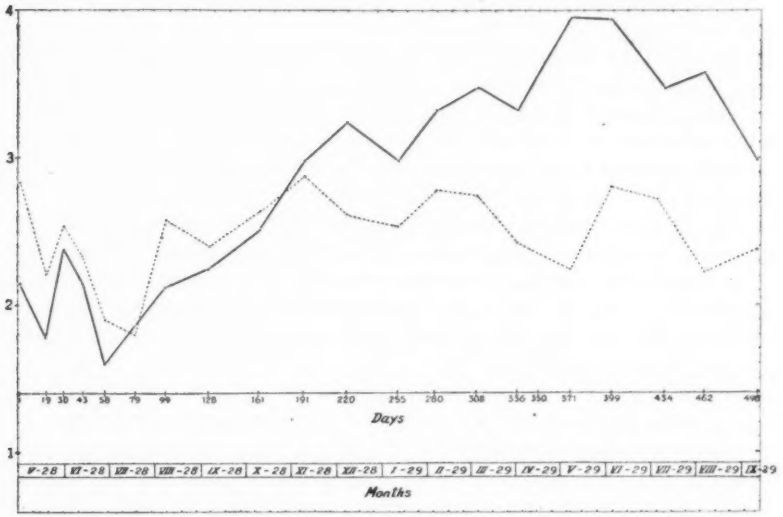

Fig. 1. The curves in graph 1 represent the number of erythrocytes per cubic millimeter in birds of both sexes from hatching to sixteen months of age. Each point is the average of two birds of each sex. The solid line represents the male, the dotted line the female. The days indicate the age of the four birds, the months the season when the readings were obtained.

The numbers of erythrocytes in millions are given in the ordinate.

bauer ruling. In the first series Hayem's solution was employed as diluting fluid but in all the later readings a 1.5 per cent solution of sodium citrate in 0.85 per cent sodium chloride was substituted: the last gave a uniformly excellent distribution.

As these birds served for other observations, they were in every instance autopsied after blood for the red counts had been collected. This study was planned to obtain records the day after hatching, twice a month thereafter to three months of age and then at intervals of one month.

Records were kept of the general condition of each bird as well as

observations on the stage of development of the primary and secondary sex characters, etc. Only completely healthy birds were used and the common origin of the flock as well as the precisely similar environment assure uniform material.

The red counts obtained in this entire series are recorded in graph 1 (fig. 1). Each point is the average of two birds of each sex. The days indicate the age of the four birds, the months the season when the readings were obtained.

The erythrocyte number in the young birds shows some fluctuation but is similar at any stage in the two sexes up to about six months of age. At this time the fowl hatched in the spring approaches maturity and the first indications of a sex dimorphism appear. The male shows an increase in the number of red cells while the female retains the juvenile level. This difference in the sexes, due to the divergence of the male from the original common level becomes progressively more distinct, and, at about nine months of age is definitely established.

This greater number of erythrocytes in the cock persists in all the observations obtained; these were continued to over sixteen months of age.

In addition to the sex dimorphism noted, some seasonal variation is suggested. Thus, in the male, the highest red counts recorded were obtained early in May and in June; the cock at this time exhibiting the greatest breeding activity.

In the female no such distinct fluctuations obtain, nor was it possible to correlate the slight variations present with the condition of the ovary.

The data presented argue for the secretory activity of the testes as causal agent in the high red count present in the cock, inasmuch as the first appearance of erythrocyte increase coincides with maturity and the highest red counts obtained with the period of greatest sexual activity.

II. The poulards: The high, male erythrocyte number should be expected in the unilaterally ovariotomised female since the activated right gonad is testis-like in character and it determines the development of male behaviour and of the male head furnishings.

The observations were therefore extended to include a series of thirty poulards having an hypertrophied right gonad and all exhibiting the male secondary sex characters.

The age of these birds at ovariotomy ranged from one day to 111 days of age. At the time the red counts were made the poulards were between two and three years of age. There is in this group no relation between the age of the bird at the time the readings were obtained and the erythrocyte number or between the age of the bird at ovariotomy and the erythrocyte number.

The poulards are therefore arranged in order of erythrocyte number in

table 1. This grouping makes it apparent at once that by far the greater number of cases show a red count equal to the male number.

III. Bilaterally ovariectomised females and capons: The number of red cells was also determined in three bilaterally ovariotomised females, one having an active testis graft, and in seventeen capons. The capon

TABLE 1

Poulards exhibiting male characters, left ovary only removed

NUMBER OF BIRD	DATE OF HATCHING	DATE OF OVARIOTOMY	DATE OF RED COUNT	NUMBER OF RED CELLS PER CUBIC MILLIMETER
1084	5-26-27	6- 3-27	7-25-29	2,370,000
892	6-30-26	9-17-26	7-27-29	2,630,000
1061	5-26-27	5-30-27	7-25-29	2,630,000
1021	5-17-27	5-22-27	7-25-29	2,670,000
1074	5-26-27	6- 2-27	7-25-29	2,680,000
1018	5- 9-27	5-22-27	7-25-29	2,720,000
821	6-16-26	7-30-26	4-16-29	2,780,000
779	4-28-26	7-16-26	4-16-29	2,795,000
1078	5-23-27	6- 2-27	7-25-29	2,920,000
788	4-28-26	7-18-26	4-16-29	3,115,000
1050	5-23-27	5-28-27	7-25-29	3,190,000
889	6-30-26	9-16-26	7-27-29	3,240,000
1058	5-26-27	5-26-27	7-25-29	3,260,000
854	6-23-26	8-13-26	4-16-29	3,355,000
873	6-30-26	9-14-26	7-27-29	3,360,000
1181	7- 1-27	8- 6-27	4-23-29	3,370,000
928	3-22-27	5- 1-27	4-23-29	3,400,000
1159	6- 7-27	7- 9-27	4-23-29	3,410,000
1091	5-23-27	6- 4-27	7-25-29	3,450,000
881	6-30-26	9-16-26	7-27-29	3,450,000
1075	5-23-27	6- 2-27	7-25-29	3,520,000
1145	6- 2-27	7- 2-27	7-23-29	3,570,000
1140	6- 7-27	6-30-27	7-23-29	3,570,000
963	4-15-27	5- 7-27	4-23-29	3,630,000
1153	6- 7-27	7- 5-27	4-23-29	3,700,000
955	4-15-27	5- 5-27	4-23-29	3,760,000
960	4-15-27	5- 6-27	4-23-29	3,780,000
1182	7- 1-27	8- 6-27	4-23-29	3,870,000
868	6-30-26	8-28-26	4-16-29	3,935,000
797	5-27-26	7-22-26	4-16-29	4,045,000

counts were obtained on various occasions; the birds were all about one year of age at the time the readings were taken and all castrated before puberty.

The figures obtained for this group are given in table 2.

This table shows the low erythrocyte count of the capon; the red counts here corresponding in general to the juvenile or female level. The bi-

laterally ovariotomized females resembling the capon in appearance also show a similar low red count. The gonadectomised female, however, having an active testis graft shows the male number of erythrocytes.

IV. General: In conclusion it may be said that in the fowl there is a definite sex dimorphism in the erythrocyte number in favour of the cock. The juvenile and gonadectomised individual of either sex and the mature female all possess a similar and relatively low red count.

The high red count of the male may be realized in the castrate by grafting testis tissue and it is characteristic of the poulard with hypertrophied right gonad.

TABLE 2

NUMBER OF BIRD	RED CELLS PER CUBIC MILLIMETER	NUMBER OF BIRD	RED CELLS PER CUBIC MILLIMETER
I. Capons			
583	1,990,000	963	2,460,000
586	2,005,000	186	2,500,000
970	2,010,000	806	2,515,000
573	2,080,000	541	2,600,000
964	2,330,000	982	2,770,000
568	2,340,000	936	2,870,000
591	2,345,000	187	3,110,000
984	2,450,000	565	3,515,000
950	2,460,000		
II. Bilaterally ovariotomised females			
With testis graft		Without testis graft	
960	3,780,000	1203	2,600,000
		1234	2,560,000

Since the erythrocyte number so clearly increases in the presence of the tesis hormone, red counts suggested themselves as a possible test for the chemically prepared male hormone. Up to the present, however, though a number of readings were obtained in capons showing satisfactory comb growth subsequent to injections of extracts of bull testes, no increase in the erythrocyte number was noted. The injections of more highly concentrated preparations may be required in order to call forth the looked for changes.

Finally it may be recorded as a suggestion that at the time of moulting the erythrocyte number appears definitely lowered and that the fluctuations in the red counts observed in the young chick coincide with the first assumption of contour feathers and the various juvenile moults.

SUMMARY

1. The erythrocyte number was determined on eighty brown leghorns, forty males and forty females, at intervals from the day after hatching to sixteen months of age.

2. A definite sex dimorphism in the number of red cells was noted confirming other observations cited.

3. The juvenile and the gonadectomised bird of either sex and the mature female all show a similar erythrocyte number.

4. The number of red cells is significantly higher in the adult male, the increase becomes apparent at the time of sexual maturity.

5. Thirty red counts carried out in poulards show that the modifications in the male direction noted after removal of the left ovary can now be extended to include the erythrocyte number which equals that of the adult male.

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METABOLISM STUDIES ON THE WILD RAT

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Received for publication June 18, 1930

In spite of the wide use of the albino rat in all kinds of physiological, bacteriological, and feeding experiments in various laboratories, and in spite of the most extended survey of the physiological and anatomical characteristics of this animal, relatively little is known with regard to its parent form, the Norway or wild rat. In his classic monograph on the rat Donaldson (1924) has recorded all the information secured on these two animals up to 1924, and in a supplementary bibliography (comprising about one-half the available titles) Drake and Heron (1930) have listed the English publications on the rat that have appeared from 1924 to 1929. The difference in the amount of knowledge concerning these two animals is evidenced by the fact that three hundred and thirty-one pages of Donaldson's monograph deal with the albino rat and but fifty-one pages with the life history and distinguishing characteristics of the Norway rat.

The gaseous metabolism of the albino rat is being studied in many laboratories, and the data obtained are being used as experimental criteria. Hence the basal metabolism of this animal is well known. Because of the profound changes in the wild rat due to domestication, and the development of the albino trait, it is important from the standpoint of comparative physiology and from the standpoint of studying the influences of captivity, change in diet, and domestication in general, to note the differences, if any, between the metabolism of the albino rat and that of the wild[1] rat. The albino rat, by careful breeding and by careful handling as regards the supply of food and the absence of infections and parasites, has been brought to a high state of perfection and has already shown the large increase in growth characteristic of animals in captivity. The wild rat also has been reported at times as being very large, but Donaldson told one of us that once, when in London, he offered an English pound for every grey rat weighing a pound (453 grams) and was unable to secure one. On the other hand, relatively large numbers of albino rats weighing over 600 grams have been secured, particularly in the colony of Dr. H. D. King at the Wistar Institute of Anatomy and Biology in Philadelphia. In any comparative

[1] In this discussion we refer to the *Mus norvegicus* alone as the wild rat.

study of the albino and the wild rat one must take into account both the modifications in the animal's organism brought about by captivity or domestication and also the possibility of a fundamental difference in the character of the wild rat, uncontaminated by any effect of captivity. These factors are best noted by measuring the wild rat immediately after it has been caught and comparing it with the albino rat. One of the earliest studies on this point was that of Chalmers Watson (1910), who compared the weights of the adrenal gland of the wild and the albino rat and showed that the wild rat has a gland weighing from two to three times as much as that of the albino rat. Watson also studied the effect of captivity on the adrenal glands of the wild rat. In his original series of wild rats (killed on receipt) the average percentage weight of the adrenal glands was 0.052 gram in the adults and 0.076 gram in the young animals. In another series of wild rats, after ten weeks of captivity, on a diet of bread soaked in skimmed milk, the percentage weight of the adrenal glands averaged 0.038 gram with the adults and 0.054 gram with the young animals. These reductions resulting from captivity are, however, in no sense comparable to the striking difference noted by Watson between the wild and the albino rat. Hence it is clear at the start that we have certain evidence of a distinct physiological difference in these two animals.

The contributions of the Nutrition Laboratory to the investigation of the metabolism of the albino rat (Benedict and MacLeod, 1929; Benedict, 1930; Horst, Mendel, and Benedict, 1930) made it incumbent upon us at least to carry out an orientation study of the metabolism of the wild rat, particularly since, in our review of the literature on the respiratory metabolism of the rat, we found no record of metabolism studies on the wild rat.

PLAN OF RESEARCH. In studying the wild rat we had three objects in view, first, to determine its basal metabolism, that is, the gaseous metabolism 24 hours after food was taken away, at the critical environment of about 28°C., and preferably during complete muscular repose. In the course of this research sufficient evidence was secured to show that the assumption that 28°C. is the critical or the optimum temperature for the wild rat is true, for experiments were made in which the temperature was not only 28°C. but also above and below this point, and the minimum metabolism was found at or near 28°C. (See pp. 674–675.) The second object of this research was to note the reaction of the wild rat to changes in environmental temperature. In a number of laboratories it has been demonstrated that the metabolism of the albino rat increases about 5 per cent with each degree (Centigrade) decrease in temperature (Goto, 1923; Terroine and Trautmann, 1927; Benedict and MacLeod, 1929; Houssay and Artundo, 1929).[2] Is the wild rat, subjected as it is in its daily life

[2] Of considerable historical interest is the research of Moleschott and Fubini (1881) who, in studying the influence of light on blinded animals, measured the carbon-

and certainly throughout the year to great changes in the temperature of its environment, less sensitive to sudden changes in temperature than the more carefully reared albino rat?

The third feature of this study was to determine the resistance of the wild rat to prolonged fasting at the optimum environmental temperature of 28°C. The experience at Yale University, New Haven, Connecticut, with albino rats that lived without food for two weeks and, in one case, for 25 days (Horst, Mendel, and Benedict, 1930) led to the belief that measurement of the gaseous exchange during fasting of itself might combine a test of the animal's resistance to fasting and a hint as to the previous storage of fat and the drafts upon body material. It is needless to say that measurements of this kind should have been supplemented by continuous determinations of the respiratory quotient and complete analyses of the rats' bodies at death. With the time at our disposal for this investigation, which was to be simply an orientation study, these observations could not be made.

In the course of the experiments it developed that the wild rat seemingly showed a marked increase in the respiratory quotient a few days before death. In other words, a "pre-mortal rise" (?) in the respiratory quotient appeared. A pre-mortal rise in nitrogen excretion has usually been noted only with the rabbit. In the experiments of Awrorow (Awrorow, 1900; Benedict, 1915) with one dog that fasted 44 days and with another dog that fasted 60 days no premortal rise in the nitrogen excretion was noted, and the calories from protein remained almost uniformly constant, amounting with one dog to 16 per cent throughout the entire fast of 44 days and with the other dog to about 11 per cent throughout the entire fast of 60 days. Therefore, although originally we had no intention of including a study of the respiratory quotient[3] in our research, this ultimately proved to be an important feature, made possible solely through the extreme accuracy of the Carpenter gas-analysis apparatus (Carpenter, 1923; Carpenter and Fox, 1926; Carpenter, Fox, and Sereque, 1929; Carpenter, Lee, and Finnerty, 1930).

Unfortunately there was a distinct omission in the experimental plan, in that the urine of the wild rats was not collected. It was impracticable

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dioxide exhalation of an albino rat at different environmental temperatures. Commenting on the marked increase in metabolism at the low temperatures, they state that their observations on the albino rat substantiate in a marked degree the law of Letellier, Lehmann, Vierordt, Colasanti, and others that with mammals and birds the carbon-dioxide excretion decreases with the increase in external temperature.

[3] The suggestion of this unusual rise in the respiratory quotient has warranted further study of this special point, and experiments are in progress at the Nutrition Laboratory at the present time to determine the course of the respiratory quotient of the albino rat with continued fasting.

to carry out this routine at the time, but in any further researches on these animals during prolonged fasting careful studies of the nitrogen output are imperatively needed.

Animals used. A number of wild rats were trapped in several different places, i.e., in a hotel basement, outdoors in an alleyway between two restaurants, in an abattoir, and in a store where poultry was kept and slaughtered. In the hotel and in the alley near the restaurants the rats could obviously secure food of a mixed nature. In the abattoir they ate exclusively animal material, and in the poultry store they had access to a considerable amount of grain but not to the viscera which were not removed after the slaughter of the poultry. Of these rats, nine were studied in our respiration apparatus. The others did not survive long enough to be measured.

Handling and care of animals. The high mortality of these rats at the beginning of the experiments was disturbing. Even before being placed inside the respiration chamber they frequently died. The explanation of this is still not available. A few of the rats were taken with snares, and although there was no external evidence of damage, undoubtedly this mode of capture contributed to their early death. Furthermore, it actually seemed at times as if the undue fright of some of these animals may have contributed to early death, and yet subsequently we were able to keep wild rats alive for many days, if not weeks, without any difficulty—animals taken in traps and not snared. The greater longevity of the rats was, however, coincidental with a suggestion of Dr. Milton O. Lee of the Harvard Medical School, that the rats be freed from lice as soon as captured. The animals were found to be covered with lice and other small parasites. Hence, acting on Doctor Lee's suggestion, we treated the rats with a solution of lysol as soon as they arrived at the Nutrition Laboratory. A solution of soap suds was made at a temperature of 38°C. To this was added lysol, representing approximately one per cent of the total amount of liquid used. The rat was dipped in this solution three times, to make sure that it was wet through to the skin, and was then left in a room at 30°C. until it became dry. Coincidental with this treatment the rats rarely died, except as a result of starvation.

The rats were housed in cages kindly loaned to us by Prof. W. E. Castle of the Bussey Institution. These cages have been described by Greenman and Duhring (1923). The rats were kept in a small room, the temperature of which could be reasonably well controlled by a steam radiator, a thermostat, and an electric fan. A continuously recording thermometer was also placed in the room and a graphic record was secured of the temperature conditions under which the animals existed for several days prior to any experiment. It was planned to hold the temperature of this room for the most part at 30°C., in accordance with previous experience at Columbia

University and Yale University. This was not always the case, however, and at times sudden changes in temperature, particularly marked falls, seemed to be accompanied by greater mortality. On the other hand, excessive rises in temperature were noted on several occasions with seemingly no untoward results.

The body weights of the rats were determined on a Chatillon spring balance that could be read to 2 grams. The rat was weighed in a container, the tare was recorded, and then the increment due to the rat noted. The rats were handled with long forceps, being picked up by the loose skin on the belly. The body weights were secured nearly every day, particularly during the periods of prolonged fasting. The animals had access to water the entire time. No attempt was made to record how much was taken or just when the animals drank prior to being weighed. The body weights, therefore, have not been corrected for any immediately preceding intake of water.

The cages during the fasting periods were supported in such a way that the feces could drop through and were not accessible to the rats for eating.

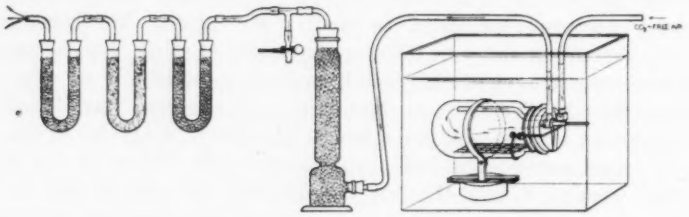

Fig. 1. Open-circuit respiration chamber for measuring the carbon-dioxide production of rats.

The food during the feeding periods was dog biscuit and a mixture of salts and grain (mostly oatmeal) kindly furnished by Professor Castle. Hence these wild rats, including the slaughter-house rats, began to subsist upon a vegetable diet immediately after capture. Since the rats did not like the dog biscuit and often refused to eat it, the oatmeal mixture alone was given toward the latter part of the research. Save when fasting, food was always accessible to the animals.

Respiration apparatus employed. At the time the experiments with the wild rats were started a multiple-chamber respiration apparatus (Benedict, 1930) was not available. The carbon-dioxide production alone was therefore measured in a simple form of respiration chamber that has been briefly described elsewhere (Benedict, 1929). The chamber (see fig. 1) consists of an ordinary one-quart glass fruit jar with a special metallic top, containing two pipes for ingoing and outgoing air. In the pipe con-

ducting the air out of the chamber is placed a small thermometer. This chamber is hermetically sealed by clamping on the top as ordinarily and immersing the jar in water in an aquarium. The details of the glass jar chamber are given in figure 2, which shows the special top removed and the small wire-mesh floor that keeps the rat off the actual bottom of the glass jar and away from any urine that might be passed. This floor contributes to quietness on the part of the animal. The bottle is held under water by being attached to a lead weight resting at the bottom of the aquarium. The water bath may be at any temperature desired, usually 28° to 29°C. An electric immersion heater in connection with a small immersion thermostat of standard make controls the temperature of the water bath. Temperatures below the room temperature are obtained by the addition of ice to the water bath.

Outdoor air, freed of carbon dioxide by being passed through a carbon-dioxide absorbent (soda-lime) is supplied to the respiration chamber by a

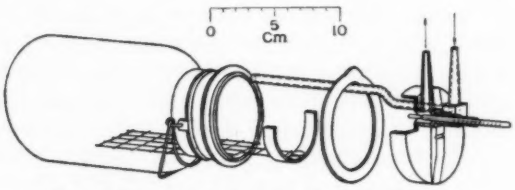

Fig. 2. Details of glass jar chamber for rats.

small rotary blower. The air leaving the chamber is passed through a calcium-chloride tower containing pumice stone drenched with sulphuric acid and then through a control U-tube containing pumice stone and sulphuric acid. The air, at this point now freed from water vapor, is next passed through two more U-tubes, the first containing soda-lime and the second pumice stone and sulphuric acid. By deflecting the air from one set of U-tubes to a second set (not shown in fig. 1), continuous periods can be obtained, as desired. These tubes are weighed every 20 or 30 minutes, and thus the weight of carbon dioxide exhaled per period is determined.

Since long experience has shown that with the rat, 24 hours after food has been taken away, the respiratory quotient is almost invariably 0.72, we have been able to compute from the carbon-dioxide production alone the heat production. We at first believed that this respiratory quotient held true not only for the first few hours of the post-absorptive condition but for many days of fasting, indeed, practically to the end of fasting. But in this research it developed that at least with a few of the wild rats there was a striking rise in the respiratory quotient, mostly a few days before death. This rise in the quotient changes the picture, in that the

caloric value of carbon dioxide must be taken not as that at 0.72 but as that at some other quotient, in order to compute the heat production accurately. In most of the experiments the respiratory quotients were not measured. When knowledge with regard to the respiratory quotient was needed, we simply adjusted the rate of ventilation so that the air leaving the chamber would contain approximately 1 per cent carbon dioxide. A sample was drawn in a sampling tube and analyzed on the Carpenter gas-analysis apparatus. Indeed, much of the last part of our research dealt with special studies of the respiratory quotient, for which purpose certain modifications in the technique were arranged. Thus, in the last few days, instead of collecting the carbon dioxide in U-tubes, the entire air leaving the chamber, containing approximately 1 per cent carbon dioxide, was deflected into a small 6-liter spirometer, such as is used in the Nutrition Laboratory for many respiration apparatus (Benedict, 1925). The total volume of air entering the spirometer for about 15 minutes was measured and from the gas analysis on the Carpenter apparatus one could compute not only the respiratory quotient but likewise the total metabolism, for a sample was drawn from the spirometer at the end of the 15 minutes and thus comprised a perfect mixture of the gas leaving the respiration chamber during the period. Successive 15-minute periods were carried out, and the total metabolism was measured in this way in consecutive periods.

Ventilation of respiration chamber. With the ordinary adult rat it has been found that a ventilation of approximately 500 to 800 cc. of air per minute will hold the content of carbon dioxide in the issuing air at about 1 per cent. This ventilation rate can be maintained in a number of ways. We have frequently employed a small, electrically driven rotary blower to suck the air from outdoors through a soda-lime bottle, to have it carbon-dioxide-free, and then to force it into the chamber. The issuing air meets with no resistance in the sulphuric acid tower and the three U-tubes, provided care is taken not to have acid accumulate in the bend of either of the acid-containing U-tubes so as to seal it. Another method, perhaps still simpler, is to suck air by means of an ordinary water suction pump through the entire system, having the air enter the chamber through a jar of soda-lime. The use of dry absorbents and absence of any liquid seals make for a free flow. Some index of the rate of ventilation is desirable. This rate can be crudely determined by the rate of bubbling of air through a wash bottle, or the air leaving the U-tubes can be passed through a meter or deflected from time to time into a small spirometer. We have found the use of a "Rotamesser" especially advantageous although by no means indispensable.

Method of introducing rats into respiration chamber. The ferocity of the wild rats made it almost dangerous to handle them without special precautions. They were therefore lifted from the cage by long forceps and

introduced into the narrow mouth of the quart fruit jar by the following procedure. A sheet brass funnel, *F* (see fig. 3), the mouth of which fitted into the top of the fruit jar, *J*, was placed in a vertical position over the fruit jar. A small flange was soldered onto the mouth of the funnel so that it rested easily in the mouth of the jar. As the rat was dropped into this funnel, a sheet of metal, *M*, was immediately placed over the open end of the funnel and the tongs were withdrawn. After a moment or two the rat usually entered the bottle of its own free will. At that moment the funnel was tilted a little to one side and a piece of sheet metal, *B*, with a slot cut in it, was slipped over the mouth of the glass bottle, so that the funnel could be removed. With the rat thus confined in the jar, the funnel could be placed at one side and then the cover, *C*, of the chamber could be adjusted by thrusting the extension of the brass pipe, *P*, that conducts

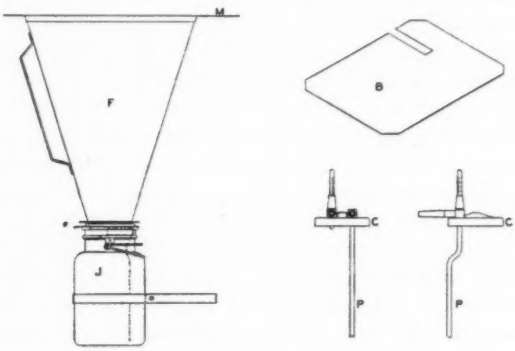

Fig. 3. Funnel arrangement for placing rat in glass jar chamber

air into the chamber, down through the slot in the sheet metal, *B*. When the cover was practically resting upon the slotted piece of sheet metal, this was slowly withdrawn and the cover was dropped into place and clamped down with the clip without the slightest difficulty. In removing the rats the cover of the jar was unclamped, the mouth of the jar placed in the cage opening, the cover slid off the jar, and the rat allowed to crawl into the cage, which it usually did after a moment's hesitation.

Discussion of results. *The basal metabolism of the wild rat.* The basal metabolism of the wild rat is of interest not only for comparison with that of the domesticated albino rat but likewise for comparison with the basal metabolism of various other animals. At the beginning of this study we were cautioned against the likelihood of our being able to make *any* determinations of basal metabolism with an animal as ferocious as a recently caged wild rat. To be sure, when taken in the traps, they were very sav-

age, and to transfer the rats from the cages in which they were ordinarily kept to the respiration chamber required much skill. They savagely attacked the forceps and resented all efforts to place them inside the chamber. However, when they were once inside the chamber, with the cover on, and when once the chamber was immersed in the water bath, the rats seemed to be resigned to the situation almost immediately and in the greater number of cases assumed the well-known, common sleeping position of the rat with the head turned under the body. To our astonishment, therefore, we secured usually in succession, four or five invariably well-agreeing periods in which the metabolism was measured, uncontaminated by *visible* activity. Graphic records of any changes of activity were not made in this orientation series of observations. Occasionally there were slight changes in body position, and it would have been much better had we recorded these graphically. Our general impression is, however, that the rats quickly became quiet and slept for a large proportion of the time. Since we had expected to lose a great many periods because of excessive activity and only occasionally obtain one with a minimum amount of movement, it was an agreeable surprise to find that in practically all of the experiments nearly every period was of actual value. This is in striking contrast to all other experiments made by the Nutrition Laboratory with any animals except the large cold-blooded animals, such as the snake. At the outset, therefore, one of the great bugbears of metabolism measurements on the wild rat, that is, inability to secure quiet periods, was not present. But although we feel certain that these basal metabolism measurements were not contaminated by visible muscular activity, in how far they were contaminated by psychic disturbances due to the change in environment and fright, we are not able to say.

The rats used in this research may be classed into two groups; first, the two rats (I and II) that were brought into the Nutrition Laboratory and studied almost immediately and that did not live long; second, those rats (III to IX) that, in accordance with the suggestion of Doctor Lee, were freed from lice and thereafter led a normal existence. This second group could be refed and brought back to their original body weight after a short fast of a day or two, and apparently continued to live with every degree of comfort, so far as is possible for a wild animal in a cage environment. In the basal metabolism study a few measurements were made with the rats of the first group, one of which (the first rat brought to the Laboratory) weighed 382 grams, a large weight for a wild rat. In table 1, in columns 5 and 6, are recorded the basal metabolism measurements on the rats of both groups, obtained at 28°C. and about 24 hours after food. Obviously nothing is known about the ages of these rats. Probably rats I and II had parasites or lice, for they were not immersed in the lysol solution. Practically all had certain abnormalities, as was found later in the autopsies (see p. 681).

The rats were placed in the chamber at about 28°C., and the carbon-dioxide production was measured in three or four successive periods of

TABLE 1

Metabolism of wild rats measured at 28° and at 20°C.

RAT AND SEX	DATE	HOURS FASTING	BODY WEIGHT	CALORIES, PER 200 GRAMS PER 24 HOURS AT 28°C.	CALORIES PER SQUARE METER PER 24 HOURS AT		INCREASE IN HEAT PRODUCTION BETWEEN 28° AND 20°C.
					28°C.	20°C.	
	1930		*grams*				*per cent*
I ♂	March 28	22	382	24.7	982		
II ♂	April 10	26	279	29.0	1,043		
Average				26.9	1,013		
III ♂	April 15	36–60	272	30.8	1,096		
IV ♀	April 17	41	172		864*	1,120	29.6†
	April 22	47	165		726*	934	28.7†
	April 25	21	172	28.8	881	1,029	16.8
	April 30	21	186	24.9	780	1,081	38.6
V ♂	April 18	25	253	29.4	1,023		
	April 23	25	251	25.5	887	1,320‡	48.8
	April 26	23	256	21.9	761	1,084	42.4
VI ♀	April 23	30	157	35.8		1,507‡	42.0
	April 23	30	157		1,061	1,453	36.9
	April 26	26	151	34.3	1,003	1,171	16.7
VII ♂	April 24	27	167	32.0	966	1,424	47.4
	April 29	28	172	28.3	870	1,270	46.0
VIII ♂	April 18	36	252	33.7	1,166		
	April 25	25	259	25.4	894	1,053	17.8
	April 30	26	254	26.3	915	1,055	15.3
IX ♀	April 24	23	292	23.9	868	1,059	22.0
	April 29	24	282	22.9	826	1,187	43.7
Average				28.3	933		32.8

* Not included in average of basal values ca. 24 hours after food.

† Included in average.

‡ This experiment at 20°C. preceded that at 28°C.; in all other cases the measurements were made first at 28°C. and immediately thereafter at 20°C.

from 20 to 30 minutes. All periods usually agreed well with each other. If they did not, because of activity in some of the periods, as many as six periods were sometimes carried out in order to secure at least three or four

sufficiently well-agreeing results to justify using an average value. From the weight of carbon dioxide absorbed and from an assumed respiratory quotient of 0.72, the heat production per 24 hours has been calculated for

TABLE 2

Changes in body weight and metabolism of wild rats during prolonged fasting

HOURS FASTING	RAT IV				RAT V				RAT VI	
	Body weight	Loss in weight	Calories per 24 hours		Body weight	Loss in weight	Calories per 24 hours		Body weight	Loss in weight
			Per 200 grams	Per square meter			Per 200 grams	Per square meter		
	grams	*per cent*			*grams*	*per cent*			*grams*	*per cent*
			28.8*	881*			29.4*	1,023*		
0	196		24.9*	780*	275		25.5*	887*	181	
							21.9*	761*		
24					254	8			166	8
48	178	9			246	11	22.5	774	158	13
72	168	14								
94	165	16								
119	159	19			200	27	22.3	717	125	31
140	152	22								
148					186	32				
161	146	25								
185	136	31	21.2	603						
Dead†	128	35			181	34			115	36
	RAT VII				RAT IX				RAT VIII	
0	184		32.0*	966*	312		23.9*	868*	268	
			28.3*	870*			22.9*	826*		
42									222	17
70	157	15			270	13			217	19
96	148	20			254	19	19.1	665	208	22
123	139	24	26.6	759	256	18				
148	138	25			238	24	20.6	703		
169	129	30								
190	117	36	22.9	613	220	29	21.1	698		
Dead†	112	39			208	33			187	30

* Basal metabolism measured at 28°C. and 24 hours after food on days *preceding* the fast.

† In all cases the dead weights were obtained within a few hours of the last live weights recorded in this table.

an average weight of 200 grams, as was done in earlier publications from this Laboratory dealing with the metabolism of the rat. Since the well-known comparison of the heat production per square meter of body surface,

instituted many years ago by Rubner, Voit, and others of the same school, still finds much vogue, we have likewise calculated the heat production per square meter of body surface. The area has been computed from the two-thirds power of the body weight in grams times the constant 9.1 suggested by Rubner (1902) and later confirmed by Giaja (1925) and Lee (1929).

Only those measurements made about 24 to 30 hours after the last food and at 28°C. have been considered as representing the basal metabolism. With these wild rats, as with the albino rat, we were uncertain as to when the last food was eaten, and about all we can say regarding the length of fasting is that the number of hours of fasting recorded in table 1 represents the time *after food was taken away from the rats.* It is more than likely that, as a result of the apprehension and terror of these rats when they were caught and carried to the Laboratory, in some instances, particularly with rats I and II, the period of fasting may have been longer than 24 hours. However, some of the observations obtained in fasts lasting longer than 24 hours did not show a profound drop in metabolism (see table 2) so that probably the results in table 1 give a rather close approximation to the basal metabolism of the wild rat.

The basal heat production per 200 grams of body weight averaged 26.9 calories per 24 hours with the two rats of the first group. With the second group of seven animals it ranged from 21.9 to 35.8 calories, but averaged 28.3 calories in practical agreement (circa 5 per cent) with the average for the first two rats. This agreement is unusual, in view of the fact that the first two rats were distinctly heavy animals and the second group contained at least three with a lower weight than the average of the first group. In general, therefore, it may be concluded that the wild rat has a basal energy output per 200 grams per 24 hours of about 27 or 28 calories.

Without discussing whether there is a causative relationship between the surface area and heat loss, we present our results computed on this basis simply as representing the heat production referred to the nearest approximation to the body surface computed in using the Rubner formula mentioned above. With the first two rats the heat production averaged 1013 calories per square meter of surface area per 24 hours. The second group, with which the measurements were by far the better because the animals were not annoyed by parasites and had become accustomed to the laboratory environment, shows striking results. In the first place, on the several (not consecutive) days on which they were measured, the metabolism invariably fell off, both per 200 grams of body weight and per square meter of body surface. On the first day the heat production per square meter of body surface was 1000 calories or over in four instances, and in at least three cases it decreased subsequently to 850 calories or below. The general average of all the basal observations secured with the second group is 933

calories. To compare the second with the first group, however, one should, strictly speaking, take only the average of the first day measurements. This average would be not far from that noted with the first group, namely, 1009 calories. The subsequent measurements made with the second group (which were not obtained upon successive days of fasting but usually at intervals of three or four days, although all of them were secured when the rats had been about 24 hours without food) show clearly the effect of the restraint in the laboratory or the difference in psychic state, if one will, and the adjustment to the environment. The wild rat has, therefore, a tendency to assume more the nature of the domesticated rat, as the period of captivity lengthens.[4] We feel confident that this difference in the basal metabolism on the first and the third days is not to be accounted for by muscular activity at the time of measurement. The rats were no quieter in subsequent experiments than they were in the first experiment. Hence the lower level of metabolism is clearly a condition imposed upon the wild rat by the experience of living in a laboratory at an environmental temperature of about 28°C.

Of special interest is the comparison of these metabolism values for the wild rat with those for the albino rat. For purposes of discussion we will confine ourselves to those researches in which the Nutrition Laboratory has recently participated (Benedict and MacLeod, 1929; Horst, Mendel, and Benedict, 1930), although fully cognizant of the importance of researches on rats that have been carried out in other laboratories. The experiments with rats at Columbia University brought out the influence of sex and particularly the influence of age. The age of these wild rats is not known. The number of animals in the total series is relatively so small and the body weights of the males and females differ so greatly that we do not feel there is any sex difference clearly shown. Rat IV, a female having a weight of 170 grams, was obviously younger than rat IX, a female of 290 grams. To compare the metabolism of these two rats alone on the basis of age would hardly be justifiable. Benedict and MacLeod concluded from their observations on female rats that the metabolism of the albino female rat at 28°C. is approximately 720 calories per square meter of body surface per 24 hours and that of male rats 14 months of age or under about 800 calories. The average of 720 and 800 calories is 760 calories. In comparison with this average the wild rats have a measurably higher metabolism, for the average of the first day's measurements is not far from 1010 calories, the average of all the measurements with the second group is 933

[4] Wu and Chen (1929) found that the basal metabolism of vegetarian rats was slightly lower than that of omnivorous rats. One might expect that our wild rats caught in the poultry house had more of a vegetarian diet than the other rats and that the vegetable diet preceding and during captivity might have played a rôle, but we have too few data on this point to draw any conclusion.

calories, and the average of the last basal values secured is somewhat under 900 calories. *The wild rat, therefore, certainly shortly after being taken into captivity, has a basal metabolism at 28°C. considerably higher than that of the albino rat.*

In the short series of fasting albino rats studied by Horst, Mendel, and Benedict, a certain amount of basal data was likewise obtained. With adult females living at 26°C. but measured at 28°C., the basal metabolism was found to be from 600 to 700 calories per square meter of body surface per 24 hours. This accentuates even more the difference between the wild and the albino rat. Probably the difference cannot be expressed quantitatively, and a simple statement that the wild rat has a much higher basal metabolism than the albino rat should suffice. That this higher metabolism is due to visible muscular activity is not true. The tendency for the wild rats to show lower values as the confinement is prolonged might hint that with a longer period of captivity the basal metabolism would possibly approach that of the albino rat. This is a problem that needs a definite solution.

Reaction of the wild rat to changes in environmental temperature. Many animals show a pronounced reaction to environmental temperature, as for example, the dog and the guinea pig, with which Rubner (1902) found an almost quantitative relationship between the metabolism and the temperature. Other animals, such as the sheep (Benedict and Ritzman, 1930) and the goose (unpublished experiments in the Nutrition Laboratory), show very slight changes in metabolism with different temperatures. Whether this is due exclusively to the better protection afforded by the wool and feathers is still debatable. The normal albino rat has been found in a number of laboratories to react strikingly to a cold temperature, its metabolism increasing 5 per cent with each degree (C.) decrease below the critical temperature (Goto, 1923; Terroine and Trautmann, 1927; Benedict and MacLeod, 1929; Houssay and Artundo, 1929). Does the wild rat react as the albino rat to pronounced changes in environmental temperature? Assuming that the critical temperature for the wild rat is 28°C. or thereabouts, we made a number of experiments to note the reaction of the wild rat to a sudden change in temperature. Most of the measurements were made, first, at 28° C. When the basal metabolism at 28°C. had been well established, the bath temperature was suddenly lowered to 20°C., and after the transition period had passed, the basal metabolism was again measured at this level. In two instances the measurements were made first at 20°C., and then at 28°C., and on one or two occasions the experiment began at 28°C., the temperature was then lowered to 20°C., and finally brought back again to 28°C. Practically all of the experiments were made about 24 to 30 hours after food was taken away. In two instances the experiments began as late as 41 to 47 hours after food was withheld.

The results of these experiments are reported also in table 1 and in figure 4. In figure 4 the abscissae represent the environmental temperature and the ordinates the calories per square meter of body surface per 24 hours. On two of the curves have been marked the number of hours that

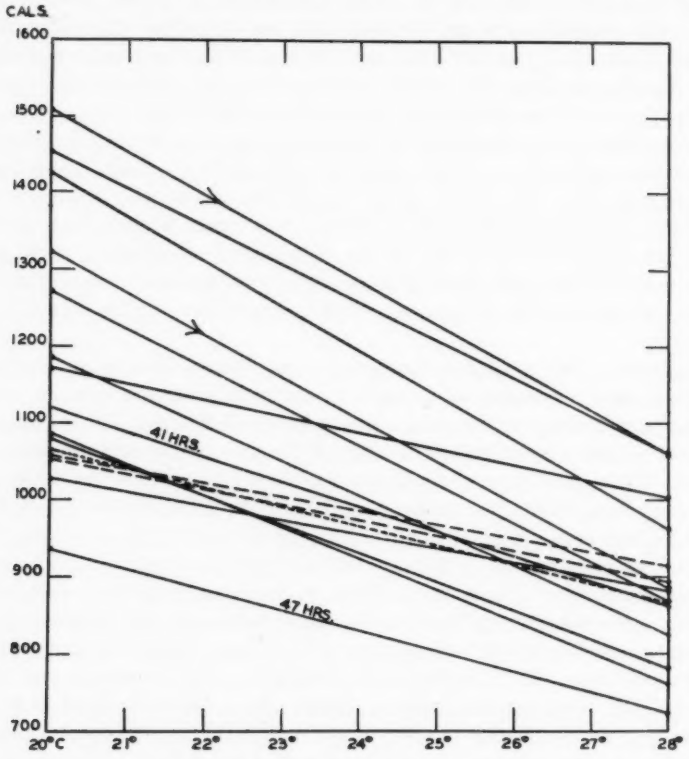

Fig. 4. Influence of environmental temperature upon heat production per square meter of body surface per 24 hours of wild rats.

The metabolism was measured, first, at 28°C. and then at 20°C., except in two instances, indicated by arrows on the curves, when the procedure was reversed. In two cases the rats were 41 and 47 hours without food (indicated on the chart) and in all other cases about 24 hours without food.

the rat had been without food, 41 and 47 hours, respectively. In all other cases the rats had fasted from 21 to 30 hours, as shown in table 1. In those two cases where the rats were changed from the lower to the higher temperature during the experiment, an arrow head (see fig. 4) points the direction on the curve. In all other cases the experiment began at the

warmer temperature. Inspection of table 1 and figure 4 makes it clear that in all cases there is a pronounced reaction to the temperature of the environment, the cold environment producing a much higher metabolism. It is not possible to state, however, that in the cold environment the animals had exactly the same degree of repose as in the warm environment. They were usually somewhat more active,[5] although it is hard to conceive of their activity as accounting for any appreciable amount of the increase in metabolism, since in those instances where the rats were as quiet at 20°C. as at 28°C. the increase in metabolism at the low temperature was just as great as in those instances where there was more activity. We believe, therefore, that we have here, as with the albino rat, to deal with a true stimulant to metabolism due to the external cold.

Although the general slope of the curves in figure 4, from the warm to the cold temperature, is upward, in some instances the degree of inclination is much more marked than in others. It was thought at first that the greatest reaction to environmental temperature might be found with the rats that had fasted the longest, but from an inspection of these curves it would seem that those fasting 40 hours or more really did not have an unduly large increase in metabolism at the lower temperature. This suggestion of a greater reaction to the temperature after prolonged fasting was borne out by one isolated experiment, not recorded in table 1 or figure 4. Thus, with one rat on the fifth day of fasting, the temperature was changed from 28° to 20°C. During the low temperature period the animal was incessantly active, trying to escape from the chamber, without a moment's rest, and this rat seemingly had a much greater reaction to the environmental temperature than did the others.

The distribution of the percentage increases in metabolism due to the change of 8°C. is considerably scattered, as shown in the last column of table 1. Thus, the total increments varied from 15.3 to 48.8 per cent and averaged 32.8 per cent. The increase in metabolism per degree decrease in temperature therefore varied from a minimum of slightly under 2 per cent to a maximum of 6.1 per cent, averaging 4 per cent. *This increase of 4 per cent is considerably less than that of 5 per cent observed with the albino rat in several laboratories.* In the Benedict and MacLeod (1929) series it was found that between 28° and 10°C. there was an increase in the heat production of albino rats per square meter of body surface per 24 hours of 51 calories for each degree that the temperature was lowered. On the assumption that the rat has an *average* metabolism of 1000 calories per square meter of body surface, it was concluded that this increase of 51 calories represents a change of 5.1 per cent. If, however, one makes the calculation upon the basis of the measured minimum metabolism of these albino

[5] The measurements of Benedict and MacLeod were not greatly complicated by marked differences in activity.

rats, that is, that actually found at about 28°C., or essentially 880 calories, the change would be not 5.1 but nearly 6 per cent. The difference between the 4 per cent increase found with the wild rat and the 6 per cent increase noted with the albino rat is, in spite of the paucity of data with the wild rats, probably significant.[6] In any event, it is clear that the wild rat is likewise susceptible to changes in environmental temperature, although not so pronouncedly as is the albino rat. When one considers how frequently the wild rat in its normal life is outdoors in the extreme cold and how often it is found around the wharves swimming in ice-cold water, it is surprising that its reaction to environmental temperature is so striking.

In a few instances when the same rat was studied repeatedly a few days apart, there was no indication of any change in the reaction to the environment. One of the rats showed its highest reaction in the fourth experiment, so there is no hint of an accommodation to the cold environment. A temperature of 20°C. is not considered in experimental laboratories as a cold environment. In fact, many rat colonies would exist at this or even a lower temperature. These considerations show that the wild rat has an extraordinary susceptibility to its environmental temperature and that the environmental temperature must play a great rôle in its food needs. This conclusion emphasizes again the necessity for knowing the temperature of the environment in rat colonies and the necessity for taking this into consideration in a study of the food needs and probably of the growth curves of rats.

Resistance of the wild rat to prolonged fasting. One of the studies made with albino rats has been to note their resistance to fasting. It is impossible to make determinations beforehand of the amount of body fat deposited in these animals, although one could analyze litter mates for control. In general, however, it seems as if a study of the resistance of the wild rat to fasting would give some hint as to the storage of body fat. Accordingly we subjected six rats to fasting prolonged until death. Our original plan was to make metabolism measurements daily (two rats each day) as the fast progressed, but the illness of one of us prevented this. Certain measurements were, however, kindly made for us by Dr. A. J. Szarka from the laboratory of Professor Verzár in Debreczen, Hungary, who was studying at the Nutrition Laboratory at the time. All the rats had had their basal metabolism measured before the fasting began, and in three instances metabolism measurements were fortunately made a rela-

[6] In the wild rat experiments the temperature difference was 8°C., i.e., from 20 to 28°C. In the experiments with albino rats the cold temperature was much lower. Since all the evidence points towards a fairly linear relationship between the temperature of the surroundings and the heat production, we believe that this comparison is valid, even if the temperature difference was somewhat smaller in the case of the wild rat.

tively few hours before death, so that the extremes in condition were studied.

The changes in body weight during the fasts, the percentages of the initial weight lost, the heat production per 200 grams of body weight per 24 hours, and the heat production per square meter of body surface per 24 hours are recorded in table 2. Prior to the fasts these rats had been freed from lice and had been receiving the oatmeal diet. They had become accustomed in a few days to laboratory existence and were apparently doing well and practically maintaining their body weight. It is impossible with wild rats, however, to know exactly when feeding ceases; hence the length of time since food was removed (reported in table 2) has been considered as representing the number of hours of fasting.

Of perhaps most significance is the loss in body weight as a result of fasting. Obviously the total loss in weight would be in large part determined by the initial weight itself. Thus, rat IX, having the largest initial weight (312 gm.), had the greatest loss in eight days, 104 grams. Rats V and VIII, with nearly the same body weight, had essentially the same loss, and the smallest losses were experienced by rats IV and VI, whose initial body weights were likewise small. The percentage loss at the time of death was practically uniform, ranging only from 30 to 39 per cent.

Three of these wild rats died on the eighth, one on the sixth, and two on the fifth day. Of the four albino rats studied by Horst, Mendel, and Benedict, which fasted at 26°C., one survived 9 days, two lived for 15 days or more, and one for 25 days. Of four albino rats fasting at 16°C., two lived practically 10 days, one nearly 13 days, and one died after about six and one-half days. The albino rats, therefore, both those fasting at 26°C. and those at the much colder temperature of 16°C. (at which the metabolism was considerably increased), outlived by a number of days the length of life experienced by the wild rat fasting under more ideal conditions at about 28° or 30°C. The loss in weight of the wild rats, which varied from 30 to 39 per cent, was really, however, somewhat greater than the loss in weight of the albino rats, which lost more nearly 25 or 26 per cent after they had been fasting for a corresponding length of time (from 6 to 8 days). Hence, *although the wild rat did not live as long, its loss in weight was much greater per day than was that of the albino rat maintained under essentially the same conditions.* Therefore we would expect to find a considerable difference in the metabolism of the wild rat as compared with the metabolism of the albino rat.

We have already pointed out (see pp. 674–675) that the metabolism of the wild rat is measurably higher, on the whole, than that of the albino rat when measured under basal conditions. How does the metabolism of the wild rat compare with that of the albino rat toward the end of fasting? Our observations on the wild rats during the progress of their fasts are

unfortunately meager, consisting of only one measurement with rat IV, two with rats V and VII, and three with rat IX. Fortunately, however, most of these observations were made toward the end of the fasting. In the discussion of the basal values found with these wild rats it was pointed out that there was a tendency for the metabolism to become lower with repeated experiments. Therefore, for the purpose of this particular comparison we can use not only the *first* basal value (which represents more nearly the metabolism before the rat has become acclimatized to its new environment) but also the last basal values, if more than one basal value was obtained. As already stated, in the calculation of the heat production from the carbon-dioxide production a respiratory quotient of 0.72 has been assumed. Frequently, especially toward the end of the fasting, respiratory quotients were found measurably higher than 0.72, at times as high as 0.80, and it is a serious question whether a respiratory quotient of 0.72 can properly be used and the carbon dioxide be considered to have the caloric value for this quotient, or whether there was a greatly increased protein disintegration with a somewhat different caloric value for carbon dioxide. But even if allowance is not made for any such difference in quotient, the maximum error in the heat calculations would be perhaps 8 or 10 per cent, which is nothing when compared to the magnitude of the decrease in heat production noted in most of the fasts of the wild rats.

Examination of the values in table 2 shows that there is a definite decrease in the heat production of wild rats with prolonged fasting. The one seeming exception is rat V, whose metabolism underwent practically no change up to the fifth day of the fast, if the lowest basal value before the fast is used for comparison. This rat's metabolism, however, was not measured so near the approach of death as was the case with the other rats. With all the rats the heat production per 200 grams of body weight was consistently not far from 20 to 22 calories on the last day of fasting. This is surprising in view of the differences in the body weights of these animals, for the initial weights ranged from 184 to 312 grams and the final weights (on days of metabolism measurements) from 117 to 220 grams. The conclusion is that wild rats, when approaching death from starvation, have a strikingly uniform metabolism of about 21 calories per 200 grams of body weight per 24 hours, irrespective of size.

The constancy in heat production toward the end of the fast is greater per unit of body weight than it is per unit of surface area. Rats IV and VII had a heat production per square meter of body surface per 24 hours as low as 603 and 613 calories. Rat IX produced more nearly 700 calories. Since the moribund albino rat has been found to have a distinctly low metabolism (Benedict and MacLeod, 1929), it is surprising that the measurements with these wild rats are as high as they are. One would expect that at least part of the excessive activity and vitality of the wild rat would

have been rapidly dissipated by the fasting. With the four albino rats studied by Horst, Mendel, and Benedict, the basal metabolism at 28°C. was at a considerably lower level than that noted with these wild rats, and after they had fasted 8 days or approximately the same length of time as the wild rats, their heat production per square meter of body surface per 24 hours averaged 510 calories. The fasting metabolism of the albino rats was therefore at a distinctly lower level than that of the wild rats. On the other hand, the wild rats were on the eighth day of fasting much nearer death than were the albino rats, for which reason one would have expected lower values with the wild rat. The albino rats in the last measurements prior to death showed values of 488, 428, 418 and 556 calories, the last value being obtained on the seventh day and the others on the sixteenth, fifteenth, and twenty-fifth days, respectively, of fasting.

Thus, by every comparison the metabolism of the wild rat is on a distinctly higher plane than that of the domesticated, albino rat. It is clear that the length of fast would normally be considerably shorter, owing somewhat to this higher metabolic level. It is by no means clear, however, that the difference in length of life of the wild rat (between seven or eight days) and that of the albino rat (more nearly 16 to 18 days) is at all commensurate with the difference in the metabolic level. Undoubtedly there was a greater storage of fat in the albino rat. This factor could not be studied in these animals, but the autopsies on both the albino and the wild rats showed that there was no visible fat left at the time of death.

AUTOPSIES.[7] As a result of our experience in an earlier study of rats (Benedict and MacLeod, 1929), most of the fasting rats were dissected after they died. In addition, some recently caught, but not starving rats were also dissected in order to have a basis for comparison. It was found that fasting caused a reduction in the size of the organs and that this reduction was the more pronounced, the longer the period of fasting. Actual weights of the organs were not obtained. The most striking difference was noted in the fat content of the body, all visible fat disappearing in the first few days of fasting. The testicles, the funiculus spermaticus, and the region around the kidneys seem to conserve longest some residual of visible fat, but even these visible traces disappear later on. With one wild rat killed immediately after capture there was a considerable stratum of fatty tissue in the subcutis, the mesenterium, the mediastinum, and the omentum. In two other cases of recently caught rats a much smaller fat reserve was found than would be expected with the average albino rat living under the usual laboratory conditions. In 80 per cent of all the rats dissected pathological changes were found, especially in the liver, presenting themselves as white spots. Tapeworm cysts in the liver were noted in

[7] We wish to express our thanks to Dr. E. E. Tyzzer of the Harvard Medical School for his assistance in making these autopsies.

about 30 per cent of the rats. These pathological manifestations had the character of a chronic malady and although they cannot be considered as the immediate cause of the death of the fasting rats, they may have contributed to the lowered resistance of these rats to fasting. This assumption is supported by the conclusion of Horst, Mendel and Benedict (1930) that the longevity of their fasting albino rats might be in large part explained by the fact that the rats were free from structural defects and both internal and external parasites.

SUMMARY AND CONCLUSIONS

Numerous measurements of the basal metabolism of the albino rat have been made. They are not wholly uniform. Even in the researches in which the Nutrition Laboratory has coöperated measurable differences in the basal metabolism of this animal have been noted. Thus, Horst, Mendel, and Benedict conclude that the basal metabolism of adult female, albino rats, living at an environmental temperature of 26°C. but measured at 28°C., about 24 hours after food, is from 600 to 700 calories per square meter of body surface per 24 hours. Benedict and MacLeod conclude that a standard value for female albino rats would be 720 calories and for male rats 800 calories. In our series of wild rats the basal metabolism at 28°C. was in all cases distinctly high. The average of all the basal measurements is 942 calories and the average of those obtained only on the first day of measurement is 1010 calories. Here it is a question whether domestication has a rapid and pronounced influence, or whether it is a matter merely of adjustment of the wild rat to a new environment with less fright. Our own belief is that the terror did not obtain, even in the first day's measurements, for the extraordinarily quiet repose of these animals astonished us. The basal heat production of the wild rats per 200 grams of body weight per 24 hours was likewise higher in the first measurement than in subsequent measurements, but averaged 27 or 28 calories as compared with an average of about 20 calories found with the albino rats measured at 28°C. On either basis of computation, therefore, the wild rat has a perceptibly higher metabolism than the albino rat.

The reaction of the wild rat to changes in environmental temperature is seemingly not so pronounced as it is with the albino rat. This may be a resultant of the resistance established by the wild rat against the widely varying environmental temperatures experienced in its normal wild life. Thus, the increase in the wild rat's metabolism averages 4 per cent per degree (Centigrade) decrease in temperature between 28° and 20°C., whereas the albino rat shows an average increase of nearly 6 per cent.

The wild rat's resistance to fasting is notably low. Even although the wild rats had been allowed several days to become accustomed to the laboratory environment and had been fed so as to maintain their body

weight, their length of life during fasting at 28° or 30°C. was, roughly speaking, one-half or less than one-half that of the relatively few fasting albino rats studied by the Nutrition Laboratory. This lower resistance to fasting may be to some extent a counterpart of the high metabolism, for even when death was approaching, the metabolism of the wild rats was at a higher level than that of the albino rats. The wild rats also lost from 30 to 39 per cent of their body weight before death, as compared with a loss of from 25 to 26 per cent noted with albino rats fasting the same length of time. Thus, the wild rat's reserves are drawn upon more rapidly by the high metabolic rate and hence are more quickly exhausted. The high level of metabolism is, however, nowhere near commensurate with the low resistance to fasting.

The albino rat, as a result of careful selection, breeding, protection, freedom from parasites, and a diet carefully established by man from laboratory standards, seemingly has succeeded in laying on a reserve against fasting that enables a much longer period of life without food. It has been thought that possibly the albino rat, because of its artificial environment and extreme pampering, might appropriately be termed a "zoölogical exotic." But these comparisons seem to disprove this, and one is led to believe that the intervention of man in the care, feeding, living conditions, and general domestication of the albino rat has been of direct ecological value to this animal.

This study of the wild rat is of special interest in connection with the larger study of the entire zoölogical range of animals undertaken by the Nutrition Laboratory, in which thus far material contributions have been made to knowledge concerning the metabolism of the albino rat, the ring dove, the common pigeon, wild birds and geese, sheep, steers, and cold-blooded animals (unpublished experiments with large serpents and tortoises). The metabolism of these various animals has been measured and the minimum or optimum metabolism found. An early conception with regard to heat loss was that this was proportionate to the surface area. In the Nutrition Laboratory series of observations the smallest heat production per unit of surface area thus far noted has been with the albino rat and the largest with the cow (Benedict, 1929). From the evidence of Aszódi (1921) the white mouse has a value even lower than that found with the albino rat. The wild rat's metabolism is considerably above that of the albino rat but not far from that of dogs and humans Admittedly the previous nourishment and the previous drag upon vitality of the horde of parasites existing on these wild rats must be important factors in determining the basal level. The psychological element, or the element of ferocity, anxiety, and terror when the animals are not asleep is likewise certainly a factor to be considered, and it is highly doubtful whether one will ever secure a series of measurements on wild animals in

their native habitat. Measurements on a series of wild birds living in a large aviary in New York led the Nutrition Laboratory to believe that just in this classification of birds alone the heat production per square meter of body surface could vary enormously, specifically, from 526 to 1267 calories (Benedict and Fox, 1927). These comparisons will be particularly interesting to those who are wont to consider the metabolism per unit of surface area as constant for all warm-blooded animals. It is obvious that further measurements are necessary of the basal metabolism of animals of various sizes, including particularly observations on body composition and nature of the integument and fur covering. This study of the wild rat, therefore, can be looked upon merely as a contribution to the larger problem of heat loss and its relation to the size, chemical composition, and zoölogical classification of the animal.

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SEASONAL VARIATIONS IN SURVIVAL AFTER ADRENALECTOMY

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Received for publication July 7, 1930

Although it appears that the cortico-adrenal tissues are quite essential to life, under some conditions significantly extended survivals after removal of the adrenal glands have been observed. The occurrence of oestrus and also of pregnancy in dogs (Rogoff and Stewart, 1927, 1928a) and the forcible administration of fluids (Marine and Baumann, 1927; Zwemer, 1927), are noteworthy examples in which death following adrenalectomy, although eventually certain, is postponed many days or even weeks. Frequent claims have also been made that extracts of the gland may prolong life (Rogoff and Stewart, 1928b, 1929; Hartman *et al.*, 1928). The recent remarkable results of Swingle and Pfiffner (1929, 1930) afford exceedingly bright promise in this direction, and indicate indeed that a very potent cortico-adrenal hormone has at last been secured.

In the course of an investigation of neural and hormonal effects on hibernation in the North American marmot (*Arctomys monax*—the common groundhog or woodchuck), it was at first noted that this animal did not withstand adrenal removal well, and often succumbed a few days after operation. No particular reason was apparent for the extremely brief survivals. With the possibility in mind that the marked seasonal variations in activity that are observed in the marmot might be involved, it was decided to extend the observations on adrenal insufficiency in this type throughout the year.

The animals were trapped in various ways, and only uninjured and vigorous specimens were used. The difficulty and danger in handling the animals precluded a very extensive series of observations being made. It was found that when kept in a well-constructed den the normal animals would survive indefinitely; thus stock animals were able to be employed in the winter, when hibernation cut off the ordinary sources of supply. Adrenalectomy is a simple operation in the marmot, and bilateral removal at one sitting, usually occupying from 10 to 20 minutes, was therefore practised. The survival periods are given in table 1.

It will be readily observed that the animals fall into two distinct groupings. In one group, those animals operated on during the summer months

of the year, the range of survival was very small—usually from 2 to 10 days only. No. 105 had been subjected to subtotal adrenalectomy some months previously and had lived through the winter; it survived 28 days after completing the adrenal removal. In the second group, comprising animals which were adrenalectomized during the winter, the survival periods were

TABLE 1

Summer and winter survival periods of adrenalectomized marmots (Arctomys monax)

ANIMAL NUMBER	SEX	WEIGHT BEFORE OPERATION	WEIGHT AT DEATH	DATE OF OPERATION	DATE OF DEATH	SURVIVAL AFTER OPERATION
		kgm.	*kgm.*			*days*
91	M.	0.9	0.9	7/ 2/29	7/ 6/29	4
92	F.	2.0	1.9	7/ 2/29	7/ 4/29	2
94	M.	1.3		7/23/29	7/24/29	1¼
95	F.	1.2		7/23/29	7/27/29	4
96	M.	2.9	2.8	8/ 1/29	8/ 4/29	3
100	M.	1.9		11/19/29	12/ 1/29	2
101	M.	2.8		11/30/29	2/28/30	90
102	M.	2.2	1.8	12/ 7/29	4/14/30	128
103	M.	1.9	1.4	1/ 8/30	2/10/30	33
110	M.	2.3	1.8	1/11/30	4/13/30	93
112	M.	2.6	2.1	2/20/30	4/ 1/30	40
113	F.	2.6	2.0	3/19/30	4/23/30	35
114	M.	2.2	1.6	3/19/30	4/17/30	29
116	F.	2.4	2.2	5/10/30	5/13/30	3
117	M.	2.0	1.2	5/10/30	5/15/30	5
120	M.	2.2	2.1	5/13/30	5/15/30	2
109*	M.	1.6	1.4	5/13/30	5/19/30	6
121	M.	1.5	1.3	5/16/30	5/21/30	4¾
105†	F.	1.6	1.4	5/16/30	6/13/30	28
106†	M.	1.2	1.2	5/16/30	5/19/30	2¾
122	M.	2.2	1.9	6/ 9/30	6/14/30	6
104*	F.	1.2	1.1	6/ 8/30	6/12/30	4
111*	M.	1.9	1.6	6/ 8/30	6/18/30	10
123	F.	2.5	2.3	6/17/30	6/21/30	4

* Previously adrenalectomized and survived through winter; accessory cortical glands removed on dates given.

† Subtotal adrenalectomy (about 90 per cent) previously carried out; animals lived through winter; adrenal removal completed on days indicated.

remarkably prolonged—from 29 to 128 days. It is also to be noted that the winter-operated animals, significantly, with two exceptions died in April. The two excepted animals succumbed, indeed, during exposure to severely cold weather (−15° to −20°C.), and apparently adrenal insufficiency was not the responsible factor. Omitting these cases, it will be observed that there are definitely diminishing periods of survival as the operations are carried out later in the winter season.

A few marmots were subjected to subtotal adrenalectomy in the early part of December, 1929; the whole of one gland and from two-thirds to three-quarters of the other were taken out at one operation. These animals—nos. 105 and 106 (table 1)—survived throughout the winter and into the summer of 1930 in apparently perfect health. In all cases, death soon followed the removal (in the summer) of the remaining adrenal tissue.

Accessory cortico-adrenals were found and removed in three animals which survived excision of the main glands during the winter. Following the second operation these animals all rapidly succumbed. The marmot has been found very resistant, it may be said, to other major operations.

TABLE 2

Observations on marmots during the active (summer) and dormant (winter) periods

GROUP AND DATE	RECTAL TEMPERATURE	HEART RATE	BLOOD SUGAR
	°C.		*mgm. per 100 cc.*
I			
June, July, August, 1929 *Animals active*	38.4	260	169 174 165
	36.3	210	163 157 153
	36.5	160	174 161 174
	38.2	252	148 146 169
	36.4	242	190 157 125
	36.5	228	160 137 138
	36.6	234	125 148
	(Av. 37°)	(Av. 241)	(Av. 157)
II			
January, February, March, 1930 *Animals dormant*	34.2	200	112 107
	33.5	90	96 128
	32.5	200	123 110
	32.5	180	118 120
	32.0	200	148 137
	34.0		
	(Av. 33.1°)	(Av. 174)	(Av. 120)

The very definite alignment of the data into what may be termed summer and winter survival groups indicated that varying metabolic activity of the animals was of fundamental importance and possibly responsible for the results obtained. When kept in well-heated quarters and given plenty of food in the early winter, the adrenalectomized animals were about as active as their normal fellows (all were less active than in summer) and they did not hibernate. Their blood sugar (method of Folin, 1928) and rectal temperature and pulse readings were slightly below the summer limits. When exposed to cold on the roof of the animal quarters all the operated and unoperated animals refused food and sought shelter, and soon became

torpid, although not particularly so. The condition might be termed one of partial hibernation.

Records derived from a number of animals under these circumstances are compared in table 2 with those taken from others during the summer months. About one-third of the readings were made on adrenalectomized animals; negligible differences only were found, in the animals in each group, and tabulation was therefore made together. It is nevertheless clear that all the figures are notably lower during the dormant season: the averages for deep rectal temperature are 37.0° in the summer (group I)

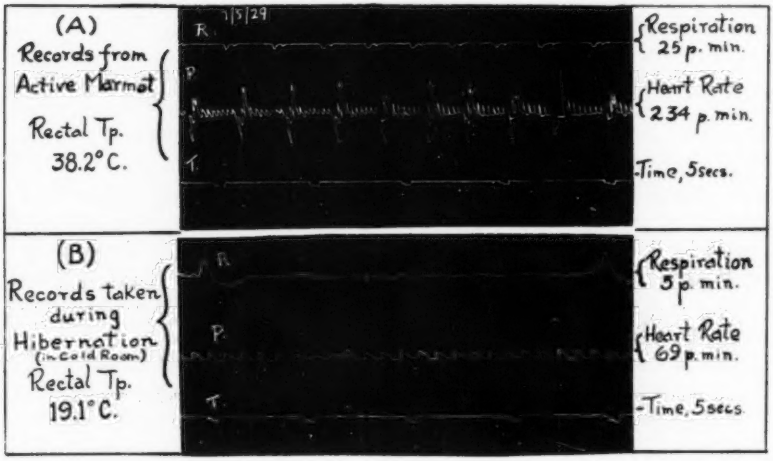

Fig. 1

against 33.1° in the winter (group II), for heart rate 241 against 174, and for blood sugar 157 in contrast to 120 mgm. per cent.

Artificial hibernation was produced in some cases by exposing marmots to freezing temperatures in a cold room. Readings which were taken from an animal by tambour-lever recording system before and after such exposure are given in figure 1, (A) and (B). The marked diminution in respiratory and pulse rates was accompanied by profound depression also of the blood glucose. In several cases, indeed, levels of 30 mgm. per cent were observed on cooling animals to about 20°C., without however the incidence of hypoglycemic convulsions (see also Britton, 1928; Tait and Britton, 1923).

These indications of a markedly altered metabolism in the marmot during hibernation or over-exposure to depressing cold are undoubtedly of significance in connection with the survival of animals adrenalectomized throughout the winter, when the needs of the (torpid) organism are con-

siderably restricted. With arrival of the normal time of awakening from winter sleep in the spring, and the greatly increased energy demands on the organism, these operated animals quickly perish. The symptoms of adrenal insufficiency do not appear during the winter or inactive season; nevertheless they are latent or merely postponed, and become evident in the warmer season. The convulsions and other terminal symptoms which were observed in animals dying in April, after having lasted throughout the winter in apparently good health, were quite similar to those seen in summer marmots which died a few days after adrenalectomy. It has been interestingly shown by other workers (Dworkin and Finney, 1927) that convulsions from insulin injections are also suppressed during artificial hibernation.

That the metabolism of the marmot may correspond during hibernation to only one-thirtieth of the amount of energy used during the period of activity was shown many years ago by Regnault and Reiset (1849). Henriques (1911) and also Pembrey (1901, 1903) have further emphasized the low respiratory quotients found in certain animals during the dormant season. More recently Gorer and Pembrey (1929) have met criticisms of their work which have been urged by other investigators. In the monograph on the marmot by Dubois (1896) a number of indications of the greatly reduced energy requirements of hibernating animals are presented at length.

Older investigations byAbelous and Langlois (1892) indicate that winter frogs survive one or two weeks after adrenal ablation, whereas summer frogs die in one or two days. Support of these observations has not, however, been forthcoming. Evidence is given by the present experiments that the adrenal glands in the mammal are intimately related to metabolism. Vigorously active animals are markedly dependent on the adrenal mechanism, and quickly perish following its removal. During periods of dormancy (hibernation) and of lowered metabolism, the adrenals appear to play a minor rôle and may be dispensed with without apparent significant effect on the organism.

SUMMARY

The marmot (*Arctomys monax*) survives adrenalectomy for two to ten days only when operated on between April and November, or during the normal active period for this hibernating type. Animals which are adrenalectomized during the winter months, when normally inactive or dormant, suffer no immediate ill effects from the operation, and may survive in good health for several months. At the usual time of awakening to activity in April such animals show severe signs of adrenal insufficiency and quickly succumb.

The blood sugar, body temperature and heart rate of the marmot were

found to be considerably lower during the normal period of dormancy than in summer. There is also a much diminished energy output during hibernation. The intimate relationship of the adrenal glands to metabolism is briefly discussed.

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THE BLOOD CALCIUM OF LAYING HENS VARIED BY THE CALCIUM INTAKE[1]

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Received for publication June 20, 1930

In a comprehensive study of the calcium cycle of the laying hen, which is now in progress at the Kentucky Agricultural Experiment Station, we have estimated that under ordinary conditions approximately 20 to 25 per cent of fertile eggs incubated do not hatch and from 5 to 10 per cent of the chicks hatched are weaklings or in some way deformed. In studying the causes affecting the hatchability of fertile eggs, we have found that hatchability is diminished until zero is reached, if a calcium carbonate supplement such as limestone, oyster shell or pure calcium carbonate is removed from certain rations fed to laying hens. Even when the hens receive sufficient sunlight or cod liver oil, a ration consisting of yellow corn, skimmilk and calcium carbonate fed ad libitum, and succulent green food given twice a week, will produce this result.

That part of this investigation which we report in this paper was planned in our search for causes or factors contributory to the non-hatchability of certain fertilized eggs. It was our purpose to determine the effect produced on the calcium content of the blood of laying hens, when the calcium carbonate supplement was omitted from a ration consisting of yellow corn, skimmilk and calcium carbonate.

With this end in view, eighteen White Leghorn hens of approximately the same age and laying conditions were selected from the experimental farm flock and placed in individual cages which were identical in size and orientation. These hens were fed for fourteen days a ration consisting of yellow corn, skimmilk and calcium carbonate ad libitum and a quantity of cod liver oil equal to three per cent of the grain fed. At the end of this period, approximately 5 cc. of blood were obtained from the larger wing vein, brachialis, of each of the eighteen hens and the percentage of calcium determined in each sample.

The hens were then divided at random into two groups of nine each and the calcium carbonate supplement was withheld from the ration fed to

[1] The investigation reported in this paper is in connection with a project of the Kentucky Agricultural Experiment Station and is published by permission of the director.

one group. A record was kept of the number of eggs laid by each hen and each egg was weighed soon after it was laid. The shell was then removed, washed free from adhering albumin with distilled water, dried at 100°C. for twelve hours, and weighed when cool.

At the end of 14 days and of 28 days from the time of withholding the calcium carbonate supplement from the diet of one lot, blood samples were obtained as previously described from each hen, calcium was determined in the separate samples and the average percentages calculated for the two lots, for the separate periods. The average number of eggs laid and the average weights of the dry shells and of the contents were calculated also.

The condition of the hens receiving the calcium carbonate supplement throughout the experiment remained good. These hens were vigorous

TABLE 1

The average calcium content of the blood serum of the hen, the average number of eggs laid and the average weight of the shells and contents for the three periods

PERIOD	AVERAGE Ca CONTENT OF BLOOD		AVERAGE NUMBER OF EGGS PER HEN		AVERAGE WEIGHT OF SHELL		AVERAGE WEIGHT OF CONTENTS	
	With $CaCO_3$	Without $CaCO_3$	With $CaCO_3$	Without $CaCO_3$	With $CaCO_3$	Without $CaCO_3$	With $CaCO_3$	Without $CaCO_3$
	mgm. per 100 cc. serum	*mgm. per 100 cc. serum*			*grams*	*grams*	*grams*	*grams*
June 6 to 20	21.3	20.9	6.3	6.9	5.0	4.8	52.3	53.2
June 21 to July 5	24.8	15.2	6.6	4.7	5.4	3.6	53.9	53.5
July 6 to July 20	21.5	16.1	9.0	1.5	5.7	3.8	56.7	47.4

and active with good appetites at the end, whereas those which received the same ration without calcium carbonate were nervous and inactive and one hen was unable to stand, and moved only when disturbed.

In other experiments it was noted that withholding a calcium carbonate supplement for a similar period of time from a grain-skimmilk ration did not cause in cocks and non-laying hens the general weakened vitality noted in this experiment. Therefore, the demand for calcium in the production of eggs and particularly egg shells is the main factor or cause for the lowered vitality of these hens.

In table 1 are given the average number of milligrams of calcium per 100 cc. of blood serum at the end of each period, the average number of eggs laid by the hens in each lot, the average weights of shells and contents of all eggs produced by the hens in each lot, for the three periods.

It will be seen that the average content of calcium in the blood serum

of the hens that received calcium carbonate was not diminished during the six weeks of the experiment, whereas in the serum of the hens that did not receive calcium carbonate the calcium content decreased from 20.9 to 16.1 mgm. in 100 cc. or about 27 per cent.

In the lot that received calcium carbonate the average weight of the contents of the eggs increased from 52.3 grams to 56.7 grams, nearly 8 per cent, while in the lot receiving the same ration without calcium carbonate it decreased from 53.2 grams to 47.4 grams, nearly 11 per cent.

The average weight of the dry shell, in the lot receiving calcium carbonate increased from 5.0 grams to 5.7 grams in six weeks, more than 12 per cent, whereas in the other lot the average weight decreased from 4.8 grams to 3.8 grams, nearly 21 per cent.

The average egg production of the hens which received calcium carbonate increased from 6.3 to 9 eggs, or 30 per cent, whereas in the other lot egg production decreased from 6.9 to 1.5 eggs, or 78 per cent, in six weeks.

TABLE 2

Estimated weight of calcium in the eggs, per hen, for the two-week-periods

PERIOD	WITH $CaCO_3$	WITHOUT $CaCO_3$
	grams	*grams*
June 6 to 20	12.5	13.2
June 21 to July 5	14.1	6.8
July 6 to July 20	20.4	2.3

In discussing the results obtained in this investigation it is evident that when a calcium carbonate supplement was removed from a grain-skimmilk-calcium carbonate ration the calcium content of the blood serum of the hens became smaller.

At the same time, egg production diminished and the weights of shell and contents became smaller. In other experiments we have shown that the hatchability of fertile eggs produced under similar conditions was diminished greatly or lost entirely. Also that with non-laying hens and with cocks there was no decrease or appreciable variation in the calcium content of the blood serum when a calcium carbonate supplement was removed from a ration similar to that used in this experiment. This indicates that the increased demand for calcium in the formation of eggs, when the supply of calcium is limited to that in yellow corn and skimmilk, causes a decrease in the amount of calcium in the blood serum, which is coincident and may be associated with the non-hatchability of certain fertile eggs. In other words, the production of eggs when the supply of calcium is inadequate disturbs the normal calcium balance of the hen.

A comparison of the average output of calcium in eggs per hen during the experiment is interesting. On the assumption that the shell contains 39 per cent of Ca and the contents 0.07 per cent, as shown by analysis made in this laboratory, the figures in table 2 were computed.

The contrast is very striking. Inasmuch as calcium must reach the egg by way of the circulation, it is not surprising that the blood content is altered in the absence of calcium carbonate.

STUDIES ON THE UTERUS

III. The Activity of the Uterine Fistula in Unanesthetized Rabbits Following Coitus and During Pseudopregnancy

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Received for publication June 25, 1930

In earlier experiments, it was noted that the activity of uterus in unmated female rabbits showed variations in intensity from time to time, as recorded by our method (1).[1] At that time it was suggested that the variations in activity might be associated with changes in a sex cycle, more specifically, perhaps, with the appearance and disappearance of heat.

The only known way to determine whether or not a doe is in heat is to offer her the service of a buck. Should the doe receive the buck, one may conclude that she was in heat. After coitus, however, there follows a series of changes ultimately resulting in some humoral change or changes (2), (3) which provoke ovulation in ten or twelve hours after coitus. Following ovulation, there ensues a period of pseudopregnancy,[2] if coitus has been sterile, during which time the effects of a functional corpus luteum become

[1] Modifications in the technic adopted by us since the time when the method was first described are as follows:

Operative: The cervical end of the uterus is secured in the caudal end of the median incision, which has been closed except for the opening through which the uterine orifices are drawn. This is done in lieu of the stab incision technic previously described. A second and important operative modification is to sew the vaginal sheath, which is exposed with the cervices of the uteri, to the recti muscles of the abdomen, instead of to the skin. The skin is drawn about the ends of the uteri as the incision is closed and anchored with several interrupted sutures into the exposed vaginal mucosa.

Balloons: The method of making balloons, as previously described, called for a "good grade of rubber cement." We now employ pure gum caoutchouc, milled and washed. This is dissolved in CCl_4. The solution is approximately 10 per cent by weight gum. We frequently dip the balloons twice, vulcanizing the gum in sulfur chloride vapor each time.

Recording system: In the description of our recording apparatus, we described a 3 mm. glass tubing on which the balloons were sealed. This was inserted into the lumen of the uterus. We now employ, in place of the glass tube no. 9 French catheter tubing of suitable length.

[2] Pregnancy, of course, cannot occur normally in these rabbits, due to the fact that in the preparation of the fistula, the genital tract is interrupted in the vagina.

manifest. About the 18th day of pseudopregnancy, or later, the doe may again come on heat, accept the service of a buck, ovulate, and undergo another period of pseudopregnancy. It was thought, therefore, that the activity of the uterus during the period of heat, and during those hours which precede ovulation, and throughout a period of pseudopregnancy would serve to enable one to correlate the activity of the uterus with the several phases of the cycle of the *mated* rabbit. Such data might serve to explain some of the physiological factors capable of modifying the activity of the uterus.

EXPERIMENTAL PROCEDURE. All of the rabbits used in this work were known to have no functional corpora lutea at the start of the experiment. This was determined by inspection of the ovaries at the time of operation, or by segregation of the does for a period of three weeks or longer. The diet consisted of hay and oats daily, supplemented by some other grain and succulent vegetables. Whole wheat, barley and bran were the grains; cabbage, beets and carrots or lettuce, the vegetables from which the choice was made. During the winter months water was supplied once or twice weekly. With such treatment, all of the animals appeared to be in excellent condition, with the exception of those whose uteri later became infected during the course of the experiment. Results obtained on these animals have been accepted only to the experimental run 4 to 6 days preceding the first sign of infection:[3] records obtained after this time have not been included in this paper.

The procedure we have used is as follows. A control run of uterine activity prior to an attempt to mate the doe was obtained. The success or failure of the attempted mating was determined by direct observation, and checked by the presence or absence of sperm in the vaginal smear. The interval after coitus at which records were obtained fall into four groups: 1, those made during a 1 to 3 hour period; 2, those made during a 5 to 8 hour period; 3, those obtained at 10 to 12 hours, or near the time of ovulation, and finally, 4, records made on subsequent days during the period of pseudopgrenancy. The records obtained through the 10 to 12 hour period we have called the activity *post coitum*; that on subsequent days, the activity associated with pseudopregnancy.

In our experiments it has been necessary to choose some way of describing the nature of our results in terms that would distinguish the several kinds of activity readily apparent to an observer. An arbitrary, but convenient and adequate grouping is noted in the legend of table 1.

[3] Uterine infection may seldom be observed from the outside of the fistula except in advanced infection. It may be detected by aspiration of pus when it is present, however.

TABLE 1

Activity in the uterine fistula post coitum

Types of activity may be designated in the following manner:

0 = quiescent condition of uterus in which no activity can be recorded with our system.

+ = feeble activity is that which with our system of recording gives records of contractions of ½ inch or slightly less.

++ = Moderate activity is that which varies between ½ inch to 2 inches, but which appears to average 1 to 1½ inch.

+++ = Marked activity is that which varies between 2 inches to 4 inches or more.

In all these classes of activity one may find rhythmical (regular) activity, or arhythmical (irregular) activity. Frequently with feeble activity one sees a sort of undulating movement, constant in rate on any one animal for a short period of time, but varying in rapidity from animal to animal.

RABBIT	DATE	ACTIVITY ANTE COITUM	ACTIVITY POST COITUM			REMARKS
			1–3 hours	5–8 hours	10–12 hours	
1a	5/5	(control)	+++	+++	+++	No coitus: See fig. 2
b	5/6	+++	+++	++		See table 2 for necropsy report: See fig. 3
2a	5/5	++	++	0	0	See table 2: coitus on 17th day; see
b	5/22	++	++	+	0	text for necropsy report
3a	2/14	+++	++	0	0	See table 2; coitus on 23rd day. In-
b	3/9		++	+	+	farct removed from uterus 3rd day of second pseudopregnancy
4	2/6	+++	++	0	0	See table 2
5	4/7	++	++	+	0	See table 2
6	4/3	+	+	0	0	Moderate activity 14 days later. See table 2
7	4/9	+	++	+	+	See table 2
8	2/12	+++	+++	++	0	See table 2
9	3/17	+++	+++	++	+	Adhesions of uterus 15 days later. No records during pseudopregnancy accepted
10	8/23/29	++	+++	+++	+	Died en route from Chicago to Philadelphia 1 week later
11	2/6	+++	+++		++	See table 2

TABLE 2

Activity of uterine fistula during pseudopregnancy

(See table 1 for legend; also for activity *post coitum*)

RABBIT	DAY 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	REMARKS
1	+							0		0		0				0			+	++				See text for necropsy report. See also fig. 3
2			0				0		0		0		0				++	Coitus 17th day; necropsy *post coitum*						
3								0				0				+		+		+				Coitus 23rd day of pseudopregnancy. See table 1
4									0															17th day pus aspirated from uterus
5																								No record; died 9th day of pseudopregnancy
6						0								++										14th day, killed this day and autopsied—see text
7			+																					Mammary gland about fistula infected, 7th day
8								0		0				+										18th day; pus aspirated from uterus
11										++														16th day pus aspirated from uterus

TABLE 3

Brief summary of tables 1 and 2

PERIOD	NUMBER OF RABBITS SHOWING TYPES OF ACTIVITY			TOTAL RABBITS
	+++	++	+	
Ante coitum	6	4	2	12
Post coitum:				
1–3 hours	5	7	1	13
5–8 hours	1	3	8	12
10–12 hours	0	1	11	12
Pseudopregnancy:				
1–9 days	0	0	7	7
10–13 days	0	1	4	5
14 days and over	0	3	2	5

RESULTS. Records following coitus have been obtained on eleven rabbits. Two of these (nos. 2, 3) again accepted coitus on the 17th and 23rd day of pseudopregnancy respectively. Thus 13 records *post coitum* have been obtained. In addition, two other rabbits accepted coitus, and have shown results that appear to agree with the other thirteen we have observed. These results are not accepted, however, because one of these animals died from unknown causes on the third day following coitus, although she was apparently in good health at the time of the experiment. The second rabbit, though observed for 26 days, gave dubious results in some instances

owing to a different type of fistula, and the consequent difficulty of inserting the balloon into the lumen of the uterus. A brief summary of the activity obtained on the eleven acceptable rabbits is given in tables 1 and 2.

Several striking features of these results stand out. A glance at table 1 reveals that preceding coitus six animals (nos. 1, 3, 4, 8, 9, 11) showed marked activity, four (nos. 2a and b, 5, 10) showed moderate activity,

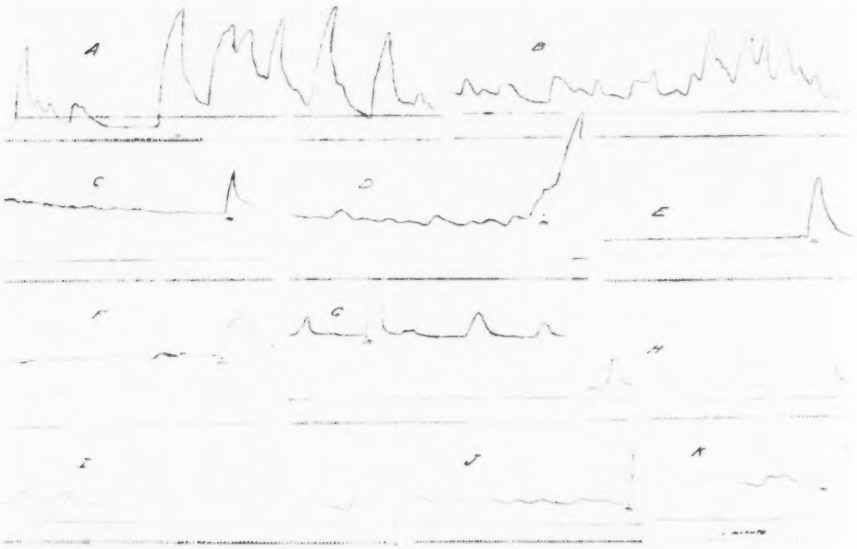

Fig. 1. Rabbit 3: Activity of uterine fistula before and after coitus, and during one complete cycle of pseudopregnancy. See tables 1 and 2.

A, normal activity before coitus; *B*, 1½ hour after coitus; *C*, 6 hours after coitus; *D*, 10 hours after coitus; *E*, 8th day of pseudopregnancy; *F*, 12th day; *G*, 16th day; *H*, 18th day; *I*, 2 hours after coitus on the 23rd day; *J*, 6 hours after coitus; *K*, 10 hours after coitus. *m*, mechanical records elicited by applying pressure to abdomen to ascertain that the system recorded freely. See table 1 for laparotomy findings in second period of pseudopregnancy. Time, 2 seconds, except where noted. ¼ original size.

and two (nos. 6, 7), feeble activity. No record *ante coitum* was made on one rabbit (no. 3b). Complete data on the type of activity found in does refusing coitus and in those accepting coitus will be published at a later time. In the 1 to 3 hour period following coitus, five rabbits (nos. 18, 9, 10, 11) showed marked activity, seven (nos. 2a and b, 3a and b, 4, 5, 7) showed moderate activity, and one (no. 6) which before coitus showed feeble activity, continued to show it here. During the 5 to 8 hour period a

striking change occurred. Only one rabbit (no. 10) showed marked activity, four (nos. 1, 8, 9, 10) moderate activity, and the remaining eight now showed feeble activity or were completely quiescent. By 10 to 12 hours the change in the activity, as contrasted with the activity before coitus, was even more striking. None showed marked activity, only one (no. 11) moderate activity, and of the remaining twelve, eleven were quiescent, or showed only feeble activity. No record was obtained on the twelfth animal (no. 1b) at this time. The summary of this analysis comprises part of table 3. (See also figs. 1 and 3.)

Fig. 2. Rabbit 1. Uterine activity in a twelve hour control experiment.
A, activity at beginning of experiment; *B*, 3 hours later; *C*, 7 hours later; *D*, 11 hours later. See figure 3 for records after coitus in this animal. Also tables 1 and 2 for data. Time, 2 seconds. ¼ original size.

The remainder of table 3 is a similar analysis of the records obtained during the period of pseudopregnancy on four animals (nos. 1, 2, 3, 6), with partially complete records on four others (nos. 4, 7, 8, 11) (see figs. 1 and 3). In all the rabbits on which records were made between days 1 to 9 *post coitum*, not one showed marked or moderate activity, but all gave evidence of only feeble activity or else were quiescent. Seven (nos. 1, 2, 3, 4, 6, 7, 8) such cases were noted. One uterus (no. 11) showed moderate activity on the 10th day of pseudopregnancy. After the 14th day an increasing activity was seen, appearing at different times in the several animals. One rabbit (no. 3), listed as having shown a relatively quiescent uterus until the 20th day, had coitus on the 23rd day without a previous control run. The activity immediately after coitus was moderate, so that one cannot say with certainty whether the activity prior to coitus was feeble or moderate. Three rabbits (nos. 1, 2, 6) were sacrificed for autopsy on the day when moderate activity first reappeared. Two of these (nos. 1, 6) which had refused coitus, had uteri which appeared grossly to be free of infection or inflammation. The uteri showed no signs of adhesions and seemed to be in perfect condition. The third animal (no. 2), it was known, had an infection

in her right uterus as a result of having lost a balloon in it six days previously, or on the 11th day of pseudopregnancy. Because it seemed unlikely that normal activity would be present in such a uterus containing a foreign body, the left uterus was used from this day till the time of her autopsy on the 17th day of pseudopregnancy. This uterus remained relatively quiescent until the 17th day when moderate activity appeared. The rabbit accepted the male on this day, and the characteristic decrease of activity *post coitum* was observed. At autopsy the left uterus proved to

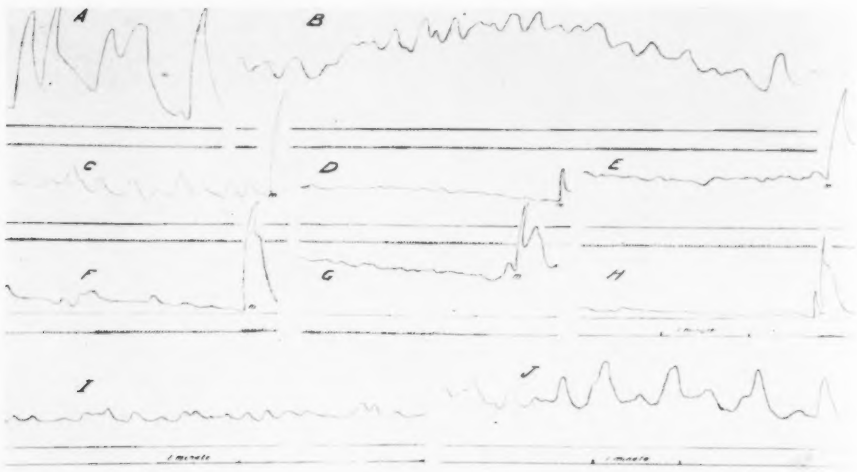

Fig. 3. Rabbit 1. Activity of uterine fistula following coitus, 16 hours after figure 2A, and during the period of pseudopregnancy which followed.

A, 1½ hours after coitus; *B*, 7 hours after coitus; *C*, 1 day after coitus; *D*, 6th day of pseudopregnancy; *E*, 8th day; *F*, 10th day; *G*, 12th day; *H*, 16th day; *I*, 19th day; *J*, 20th day. *m*, mechanical records, as in figure 1. See text for necropsy report on 20th day of pseudopregnancy. Time, 2 seconds. ¼ original size.

be free of infection; it was also determined that ovulation had not yet taken place. The return, then, to moderate activity in these three rabbits, and possibly in a fourth, may be regarded as a return to an activity analogous to that seen *ante coitum*.

Discussion. One might object that this decrease in activity following coitus is an artifact, due to the fatigue of the uteri during the 10 hour run after coitus. The following considerations make this seem unlikely. A control on rabbit 1 (fig. 2) shows that the mere fact of recording at the intervals we have chosen does not lessen the activity unless the doe has coitus, or unless some experimental procedure is resorted to. In this instance

at least, the activity remained marked for more than twelve hours, and the following day showed a post coital decrease in activity in the expected time. Moreover, in other work soon to be reported, we have obtained ample evidence that recording over a ten hour period in no way affects the uterine activity *per se*.

The uterine activity associated with the oestral cycle, as described by Keye (4), indicated that prior to, and even after ovulation in the sow, large contractions occur. This was true of the excised strip obtained from different animals at various stages of the oestral cycle. The time in the sex cycle was placed by Keye by inspection of the endometrium, and the condition of the ovaries at the time of excision of the uterus. A progressive decrease in the amplitude of contractions occurs concurrently with the formation of corpus luteum, reaching a minimum as the corpus luteum attains full growth. Thus, according to Keye, the formation and persistence of this structure exerts a profound influence on the motility of the uterus. Somewhat similar observations have been made on the white rat (5). Knaus found (6) a relative quiescence in excised uterine strips obtained from rabbits during pregnancy, as does Keye in the sow. The evidence seems to indicate, therefore, that some changes associated with the development and persistence of the corpus luteum may be important factors in modifying uterine activity.

The results we have obtained, however, indicate that there are other factors that precede any effect the corpus luteum may exert, in the rabbit at least. This becomes apparent when one considers that the decrease in activity is practically attained within 5 to 8 hours after coitus, and is nearly complete by the 10th hour. Ovulation occurs at about the 10th hour or a little later, and the corpus luteum surely does not commence to form until after this time. In the rabbits, apparently, some changes result from the stimulus of coitus, which are directly or indirectly responsible for this decrease in activity *post coitum*.

One is led to inquire, therefore, what the nature of these changes might be. Are they predominantly nervous or humoral? The rôle of a reflex element is a possibility. It seems unlikely, however, that an effect which was primarily nervous would require five or more hours to manifest itself.

Possibly some light may be thrown on the question from indirect sources. Friedman (2) has shown that a humoral mechanism is responsible for ovulation in ovarian transplants in rabbits. Fee and Parkes (3) find that removal of the hypophysis of a doe within one hour after coitus will prevent ovulation. If, on the other hand, the hypophysis be ablated sometime later than an hour after coitus ovulation proceeds as usual. One might like to know, therefore, whether the humoral changes responsible for ovulation exert an influence directly or indirectly on the uterus to initiate uterine inhibition. One might also ask whether the continued action of the agents

responsible for the initial inhibition is capable of maintaining the uterus in a quiescent condition during pseudopregnancy, or, whether it is the corpus luteum which has assumed this rôle. All these questions demand further investigation.

SUMMARY

1. Female rabbits prior to coitus are seen to exhibit marked or moderate uterine activity. In two cases of twelve, however, feeble activity preceding coitus has been observed.

2. During the ten hour period following coitus, and before the time of ovulation, the uterine activity becomes greatly decreased. The decrease in activity is seen about the 5th to the 8th hour, and is still more pronounced between the 9th to 12th hours *post coitum*.

3. During the period of pseudopregnancy, the uterus remains relatively quiescent until about the 15th to the 20th day, when activity may return.

4. The possible factors responsible for the initiation and maintenance of the quiescent condition of the uterus are considered.

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STUDIES ON THE UTERUS

IV. The Response of the Uterine Fistula of the Unanesthetised Rabbit to the Injection of Urine from Pregnant Women

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From the Department of Physiology of the University of Pennsylvania Medical School

Received for publication June 25, 1930

The finding that the activity of the uterine fistula is markedly decreased within five to eight hours following coitus (1) raised several questions as to the mechanism of this reaction. In view of the relatively long latent period it was doubted that this decrease in uterine activity, as recorded by our methods, was a direct, or indirect reflex effect of the act of coitus. Insofar as the single, intravenous injection of urine from pregnant women is capable of simulating the humoral changes following coitus in the rabbit and leads to ovulation in the injected animal some ten or more hours after the injection (2) (3) (4), it was thought that the response of the uterine fistula to the injection of such urine might yield some clue as to the nature of the processes responsible for the decrease in uterine activity following coitus.

Experimental procedures and results. Control records of the activity to the uterine fistula were obtained from 10 rabbits with the method used by us in our previous studies. It had been determined by laparotomy that the ovaries of each of these animals were free of corpora lutea. Immediately after obtaining these control records, five of the ten animals were injected intravenously with 5 cc. of urine from a pregnant woman. The remaining five rabbits were similarly injected with urine from a non-pregnant woman. The activity of the uterine fistula in each of the ten animals was then recorded at a period six to seven hours following the injection, and later, at a period ten to twelve hours after the injection. In every instance the activity was recorded for about a half-hour.

The rabbits injected with the urine from a pregnant woman all showed a decreased uterine activity by the eighth hour following the injection. Not one of the rabbits injected with urine from a non-pregnant woman gave evidence of a decrease in uterine activity. A few days later, another control record of a half hour was made on the fistula of each of the five rabbits which had been injected with urine from the non-pregnant woman. Immediately afterwards these rabbits received an injection of urine from a pregnant woman. By the eighth hour after this injection the activity of the fistulae in all five rabbits was strikingly decreased.

TABLE 1

Activity of the uterine fistula following injection of urine from non-pregnant women

Types of activity may be designated in the following manner:

0 = quiescent condition of uterus in which no activity can be recorded with our system.

+ = feeble activity is that which with our system of recording gives records of contractions of ½ inch or slightly less.

++ = Moderate activity is that which varies between ½ inch to 2 inches, but which appears to average 1 inch to 1½ inch.

+++ = Marked activity is that which varies between 2 inches to 4 inches or more.

In all these classes of activity one may find rhythmical (regular) activity, or arhythmical (irregular) activity. Frequently with feeble activity one sees a sort of undulating movement, constant in rate on any one animal for a short period of time, but varying in rapidity from animal to animal.

RABBIT	DATE	ACTIVITY OF FISTULA			
		Before injection	1-3 hours	6-7 hours	10-12 hours
1	6/9	++			
	6/12	++	++	++	++
2	6/9	++			
	6/12	++	++	++	++
3	6/12	+	+	+	++
4	6/12	++	++	+++	+++
5	6/3	+++		+++	+++

TABLE 2

Activity of uterine fistula after the injection of urine from pregnant women

RABBIT	DATE	NORMAL	AFTER INJECTION			REMARKS—48 HOURS LAPAROTOMY
			6-7 hours	10-12 hours	24-48 hours	
1	6/15	++	+	+		See table 1 for control; corpora lutea
2	6/15	++	+	0		See table 1 for control; corpora lutea
3	6/15	++	+	0		See table 1 for control; corpora hemorrhagica
4	6/15	++	+	+		See table 1 for control; corpora hemorrhagica
5	6/4	+++	+	0		See table 1 for control; corpora hemorrhagica
6	5/25	+++	+	0	+	Corpora lutea and ovulation
7	5/27	+++	0		0	Corpora lutea; ovulated
8	5/25	+++	+	+	+	Corpora lutea
9	5/25	+++	+	+	+	Corpora lutea
10	5/25	+++	+	+	+	Corpora lutea and ovulation

In table 1 is given a summary of the results of the five experiments in which urine from non-pregnant women was injected. In table 2 there appears a similar summary of the results obtained in the ten experiments in which urine from pregnant women was injected; added thereto are notes of the findings at laparotomy 48 hours after the injection.

DISCUSSION. We regard it as extremely suggestive that those changes in the ovary, and in the activity of the uterus, which are the consequences of coitus in the female rabbit can be so closely reproduced by the intravenous injection of urine from a pregnant woman. It is just because the changes in uterine activity following the injection of such urine so exactly duplicate the changes in uterine activity following coitus, that we have not submitted the records of these experiments. Yet, we have not yet the right to identify the substance, or substances, in the urines of pregnant women as the same as those that are presumably discharged into the blood or body fluids of a female rabbit after coitus (5) (6). Moreover, we dare not venture the statement that the substance responsible for the ovarian changes is the one responsible for the decrease in uterine activity. It is quite possible that the agent causing the change in uterine activity is ovarian in origin, or is liberated from some other source in response to coitus, or to the injection of urine from a pregnant woman.

SUMMARY

1. The intravenous injection of urine from a pregnant woman into female rabbits regularly decreases the activity of the uterine fistula as recorded by our methods.

2. The similar injection of urine from a non-pregnant woman leaves the activity of the uterine fistula quite unaltered, or increases it slightly.

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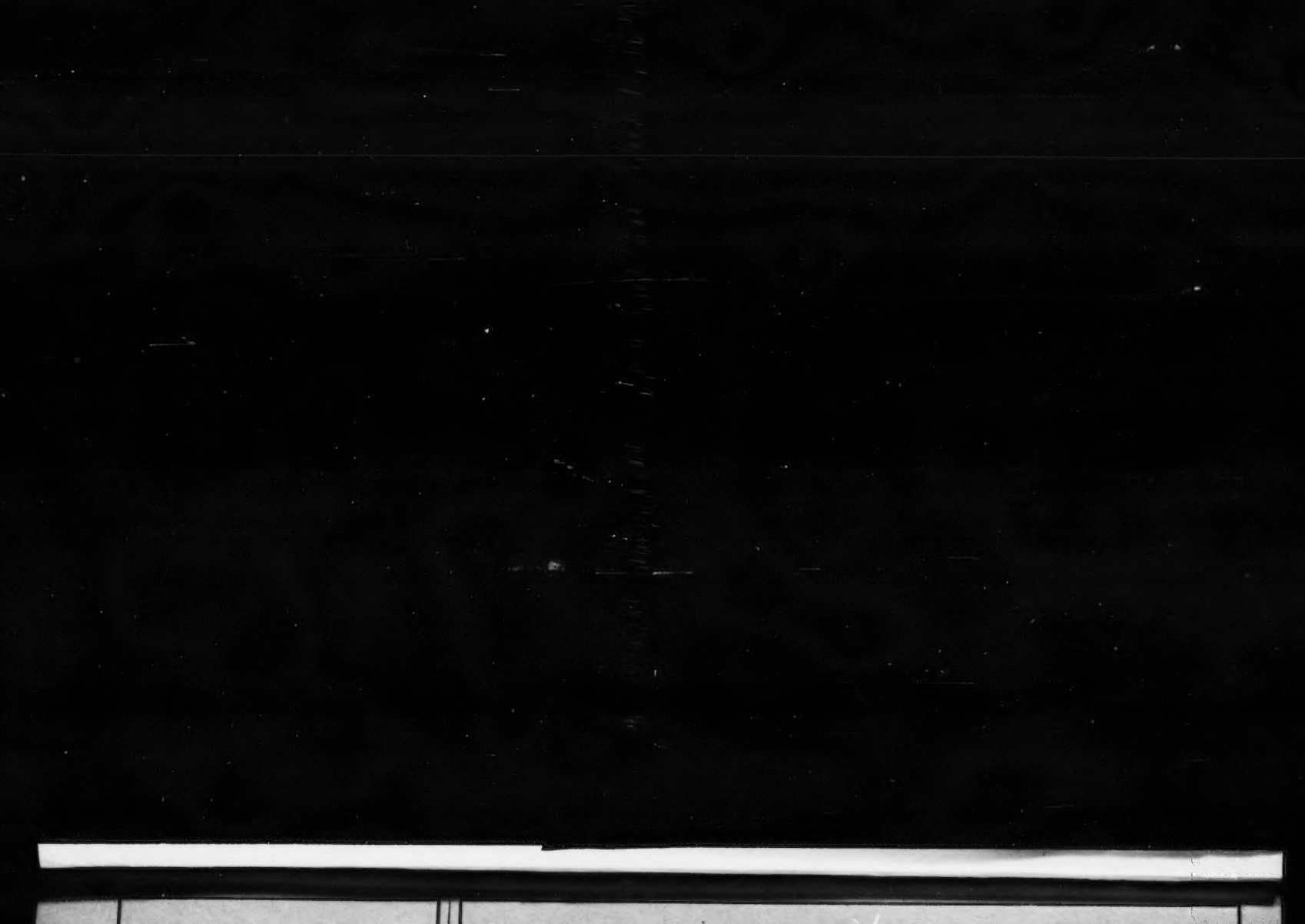
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